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14. ABSTRACT
The overall goal of this project is to understand the role of inducible inflammation-related kinase IKKi in constitutive activation of anti-apoptotic transcription factor NF-κB prostate carcinoma (PC) cells. We found that IKKi is expressed in highly malignant androgen-independent PC cells lines and in epithelial cells in benign and malignant prostate lesions. Our data provide experimental evidence that IKKi could be involved in the regulation of activity of major anti-apoptotic factor NF-κB in PC cells through a positive feedback loop. Our results also suggest that IKKi may play an important role during the transition to hormone refractory stage of PC growth via its positive effect on the nuclear translocation and activity of androgen receptor in PC cells. Taking into consideration the newly recognized association between prostate inflammation and increased risk of PC development, we extended our studies towards cross-talk between pro-inflammatory signaling mediated by IKKi and NF-kB and anti-inflammatory signaling mediated by glucocorticoid receptor (GR) in PC cells. We showed that GR functions as a tumor suppressor in prostate cells, and that inhibition of transcription factors involved in proliferation and transformation in PC cells, including NF-kB, is the major molecular mechanism of GR anti-tumor activity. As IKKi specific inhibitors are still not available, we screened several novel classes of NF-kB inhibitors for their growth-inhibitory and anti-apoptotic effects in PC cells. The results of our studies have been presented at the local and national meetings, five manuscripts have been published, one is under revision, and two are under preparation.

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Report for FY04.

Introduction

The overall goal of this project is to understand the role of inducible IKK-related kinase IKKi in constitutive activation of anti-apoptotic transcription factor NF-κB prostate carcinoma (PC) cells.

During FY04 (no cost extension) the major direction of our work was to complete the evaluation of the biological effects of IKKi overexpression in PC cells stably infected with lentiviruses harboring w.t. IKKi, and the important role of IKKi in the transition of prostate cells to androgen-independent growth. The results of our studies in 2006-2007 have been presented at the national meetings, two manuscripts are published, one is under revision, and two are under preparation. The following describes the progress made in this year.

Body

During FY04 we specifically focused on the experiments pertinent to our most important findings made in the previous years suggesting that IKKi may represent a link between inflammation and androgen receptor signaling. We continued to characterize the effect of IKKi in PC cells on basal and inducible NF-kB activity, growth, tumorigenicity and AR function.

We found that that IKKi expression in LNCaP and PC3 cells resulted in increased basal NF-kB activity measured in Luciferase assay (Fig. 4B – cells transiently transfected with w.t. IKKi, Fig. 6.A1 and 6.A2 – cells stably infected with IKKi lentivirus). IKKi-expressing cells were more sensitive to NF-kB inducers such as TNF-a, TPA, EGF, LPS, and especially IL-1 (Fig. 6.A1 and 6.A2). In addition to the experiments with exogenous kappaB reporter, we evaluated the effect of IKKi on the expression of endogenous NF-kappaB-responsive gene IkBa using RT-PCR analysis, and found that IkBa expression was induced more effectively in LNCaP-IKKi cells (Fig. 6.B1).

We confirmed that induction of IkBa phosphorylation (at Ser32/36) and p65 phosphorylation at Ser536 was increased in LNCaP-IKKi cells treated with such inducers as TNF-a and LPS (Fig.6. C1 and C2). It is well known that IkBa undergoes proteasomal degradation after phosphorylation at Ser32/36 (Ref). Thus, the effect of IKKi on basal and inducible IkBa phosphorylation was more augmented when degradation of phosphorylated IkB-a was blocked by proteasome inhibitor MG132 (Fig. 6.C1, far right lanes).

We showed that IKKi expression results in increases PC growth and tumorigenicity. Using LNCaP and PC3 clones co-expressing w.t. IKKi (Fig. 5C) and yellow fluorescent protein (YFP) to track live cells and to measure the actual number of cells/well by fluorescent plate-reader, we showed that IKKi significantly increased PC cells growth in monolayer (Fig. 5.A1. and A2). Empty-vector-expressing cells (LNCaP-V and PC3-V) were used as control. Importantly, IKKi-expressing LNCaP cells were also characterized by the increased tumorigenicity assessed by anchorage-independent growth (colony formation assay in soft agar, Fig.5. B).

We further showed that IKKi overexpression resulted in the partial nuclear localization of endogenous androgen receptor (AR) in LNCaP cells (Fig. 7). It correlated with the increased transcriptional activity of AR in LNCaP-IKKi cells growing in the normal cell culture medium (standard fetal bovine serum) and in the medium with the decreased androgen level (charcoal-stripped serum) especially when cells were activated by androgen DHT (Fig. 8 B). The results of Luciferase assay with ARE reporter have been further extended using RT-PCR analysis of the expression of androgen-dependent endogenous gene PSA (Fig. 8.C). As shown in Fig. 8.C IKKi-expressing cells were more responsive to DHT stimulation which is reflected by significantly early increase in PSA expression in comparison to cells infected with empty virus.

Most importantly, LNCaP-IKKi cells became partly androgen-independent: they grew better than LNCaP-vector transfected cells in the medium with charcoal-stripped serum. This correlated with the

spontaneous translocation/accumulation of AR in the PC nuclei (Fig. 8A).

Overall, our results suggest an important role of IKKi in the transition of prostate cells to androgen-independent growth. We are currently testing the hypothesis that IKKi may affect the level of AR phosphorylation which in turn can result in spontaneous nuclear AR translocation. For this we are using Western blotting with antibodies against phosphorylated AR.

Taking into consideration the newly recognized association of prostate inflammation and prostate cancer that offers one of the greatest opportunities for preventing malignant conversion (Platz and De Marzo, 2004,De Marzo et al, 2007) we continued to study the cross-talk between pro-inflammatory signaling mediated by IKKi and NF-kB and anti-inflammatory signaling mediated by glucocorticoid receptor (GR) in PC cells. In FY02 –FY03 we found that glucocoticoids inhibit IKKi function; we also showed that GR acts as a tumor suppressor in prostate cells. In FY04 we started to work with a unique compound (CpdA) that acts as a non-steroidal ligand of both AR and GR (Fig. 9 and data not shown). Similar to steroid hormones, CpdA induces nuclear translocation of both receptors in prostate cells. Despite of this, CpdA inhibits DNA-binding and transactivation potential of AR (data not shown). In addition, CpdA inhibits GR-mediated transactivation but induces GR trans-repression via inhibition of transcription factors, first of all, NF-kB (Fig. 9). CpdA strongly inhibits growth and induces caspase-dependent apoptosis in highly malignant PC cells in AR/GR-dependent manner (Fig. 10 and 11). Overall, our data suggest that CpdA is a unique dual-target steroid receptor modulator that has a high potential for PC therapy.

Key Research Accomplishments FY04

- The increased IKKi expression in different PC cells stably infected with IKKi-lentivirus results in increased basal and inducible NF-kB activity.
- IKKi overexpression results in increased growth and tumorigenicity of PC cells.
- Androgen receptor was partially translocated to the nucleus and constitutively activated in IKKi-expressing PC cells.
- LNCaP-IKKi cells became partially androgen-independent and could sustain the androgen ablation.
- These results suggest that IKKi plays an important role during the transition to hormone refractory stage of PC growth.
- Compound A, a non-steroidal modulator of glucocorticoid receptor that inhibits NF-kB function, inhibits growth and viability of highly malignant prostate cancer cells.

Reportable Outcomes FY04

Manuscripts:

1. Yemelyanov A., Czwornog J., Chebotaev D., Karseladze A., Kulevitch E., Yang X., Budunova I. Tumor suppressor effect of glucocorticoid receptor in prostate. Oncogene, 2007, 26:1885-1896.

2. T. Nelius, S. Filleur, A. Yemeyanov, I. Budunova, E. Shroff , Y. Mirochnik, A. Aurora, D. Veliceasa, W. Xiao, Z. Wang, and O.V. Volpert Androgen receptor targets NFkB and TSP1 to suppress prostate tumor growth in vivo. Int J Cancer. 2007, 121(5):999-1008.

3. Yemelyanov A., Czwornog J., Joshi S., Gera L., Budunova I. Compound A, a novel phyto-modulator of steroid hormone receptors, as a candidate for prostate cancer therapy. Submitted.

4. Yemelyanov A., Kobzeva V., Budunova I. Role of IKKi in prostate cancer: A link between inflammation and androgen receptor signaling. Manuscript is under preparation.

5. Gasparian A., Yemelyanov A., Chebotaev D., Kisseljov F., and I. Budunova. Targeting NF-κB in prostate carcinoma cells: comparative analysis of proteasome and IKK inhibitors. Manuscript is under preparation.

Abstracts presented at the national meetings:

1. Yemelyanov A., Kobzeva V., Budunova I. Role of IKKi in prostate cancer: A link between inflammation and androgen receptor signaling. Proceedings of AACR, 2007 (abstract # LBA-9158).

2. Budunova I. , Yemelyanov A., Gasparian A. Role of IKKs and transcription factor NF-kB in prostate tumorigenesis. P. IMPACT DOD meeting, 2007, Atlanta.

Seminars presented by P.I.

Compound A, a novel phyto-modulator of steroid hormone receptors, as a candidate for prostate cancer therapy. Tumor cell biology seminars. R. Lurie Cancer Center. NU. April, 2007.

Non-steroidal modulators of steroid hormone receptors as candidates for prostate cancer therapy. Children's Memorial Research Center, Chicago. May , 2007

Conclusions for FY04.

Our data provide experimental evidence that IKKi could be involved in the regulation of NF-kB activity in PC cells through a positive feedback loop. For example, NF-kB was constitutively activated in PC cells stably infected with w.t. IKK-expressing lentivirus. IKKi –infected cells were more responsive to different NF-kB inducers. IKKi is highly expressed in androgen-independent malignant PC cell lines. The

introduction of w.t. IKKi into androgen-dependent LNCaP prostate cells significantly increased their growth and tumorigenicity, and protected these cells from the induced apoptosis. Remarkably, IKKi expression in LNCaP cells resulted in nuclear translocation and increased transcription potential of androgen receptor (AR). Moreover, LNCaP-IKKi cells were more resistant to androgen ablation than parental LNCaP cells. Those findings suggest that IKKi may play an important role in the development of hormone refractory phase of PC growth.

Final Report for the entire funding period

Introduction

The overall goal of this project is to understand the role of inducible IKK-related kinase IKKi in constitutive activation of anti-apoptotic transcription factor NF-κB prostate carcinoma (PC) cells. The recent literature data published during the funded research period clearly indicated that the expression of this novel upstream IkappaB kinase strongly depends on inflammatory cytokines. It also became clear that there is a causative link between prostate inflammation and increased risk of PC development. Thus, we extended our research towards studies of cross-talk between pro-inflammatory signaling mediated by IKKi and NF-kB and anti-inflammatory signaling mediated by glucocorticoid receptor (GR) in PC cells. Unfortunately, small inhibitors of IKKi remain to be developed. Thus, we searched for other effective strategies of NF-kB blockage in PC cells, and tested growth inhibitory and pro-apoptotic potential of several novel compounds including highly specific IKKβ inhibitors, proteasomal inhibitors as well as dissociated ligands of GR that inhibit NF-kB.

Body:

Task 1. To define whether IKKi is an essential part of a positive feedback regulation of NF-κB in PC cells.

We found that IKKi is expressed only in highly malignant, androgen-independent PC cells DU145 and PC3 (Fig. 4 A1). We also showed that IKKi is highly inducible in PC cells by NF-κB activators such as IL-1α and TNF-α (Fig. 4.A2). TPA appeared to be less active as IKKi inducer (data not shown). Sensitivity to IKKi induction correlated well with sensitivity of specific cell line to NF-κB induction by different inducers (Gasparian et al, 2003, abstract # 4). Consistent with this, down-regulation of NF-κB activity by proteasome inhibitors and IKK inhibitor PS1145 attenuated induction of IKKi expression by NF-κB inducers (Yemelyanov et al., 2003. Abstract #3).

Using transient transfections of PC cells with w.t. IKKi (kindly provided by Dr. Mercurio, Signal Pharmaceuticals, Inc., San Diego, CA) and kinase inactive IKKi mutant, K38A (kindly provided by Dr. Maniatis, Harvard Medical School, Cambridge,MA) we confirmed that IKKi plays an important role in the maintenance of NF-kB basal activity in PC cells (Fig. 4B).

We also performed stable transfection of PC cells with high constitutive IKKi expression with IKKi d.n. construct and LNCaP cells that do not express endogenous IKKi with w.t. IKKi. Unfortunately, most of the selected clones lost the transgene expression during the passaging.

Thus, in our next cycle of experiments we generated several lentiviruses harboring empty vector, IKKi-FLAG, d.n. IKKi-FLAG, and generated stably infected PC3 and LNCaP cell lines with different IKKi status co-infected with YFP-expressing lentivirus. As shown in Fig. 5C, PC cells infected with IKKi w.t. lentiviruses, stably expressed transgenic IKKi tagged with FLAG. We tested the sensitivity of those IKKi-expressing cells to different NF-kB inducers, and showed that IKKi-expressing cells were overall more sensitive to NF-kB induction (Fig. 6A). They also had higher basal NF-kB activity than cells infected with empty virus (LNCaP-V and PC3-V respectively, Fig. 6A). In addition to the experiments with exogenous kappaB reporter, we evaluated the effect of IKKi on the expression of endogenous NF-kappaB-responsive gene IkBa using RT-PCR analysis, and found that IkBa expression was induced more effectively in LNCaP-IKKi cells than in LNCaP-V cells (Fig. 6.B1).

We also found that induction of IkBa phosphorylation (at Ser32/36) and p65 phosphorylation at Ser536 was increased in LNCaP-IKKi cells treated with such inducers as TNF-a and LPS (Fig.6. C1 and C2). It is well known that IkBa undergoes proteasomal degradation after phosphorylation at Ser32/36 (Ref). Thus, the effect of IKKi on basal and inducible IkBa phosphorylation was more augmented when

degradation of phosphorylated IkB-a was blocked by proteasome inhibitor MG132 (Fig. 6.C1, far right lanes).

Overall, these data provide the experimental evidence that IKKi could be involved in the regulation of NF-κB activity in PC cells through a positive feedback loop.

Task 2. To study the expression, subcellular localization and interaction of IKKi and its target proteins IKKβ, I-TRAF, and TRAF2 in PC cell lines and PC tumors.

Using prostate samples provided by NU prostate SPORE tissue core we performed immunostaining of more than 60 formalin-fixed paraffin-embedded samples of BPH and PCs using multiple antibodies against IKKi (four different Abs from Imgenix, Santa Cruz., Active Motif, Pro-Sci). We also performed immunostaining with different antibodies to reveal localization of potential IKKi target proteins such as IkBa (we used anti-phospho-IkBa Ab from Cell Signaling), IKKb (two different Abs from Santa Cruz and Imgenix, and Ab against phospho-IKKb from Cell Signaling), TRAF2 and I-TRAF (both from Santa Cruz) in benign and malignant prostate lesions. The staining was reviewed and scored by pathologists at Pathology Core at R. Lurie Cancer Center, who have an extensive experience in quantitative analysis of PC marker expression.

Western blot analysis of the IKKi Ab specificity revealed that the best anti-IKKi antibody was a monoclonal Ab from ProSci that gave only one specific band on Western blots when we used protein extracts from control and treated PC cells (Fig. 2). Thus, we analyzed IKKi localization using immunostaining of prostate tissues only with this antibody. The analysis of IKKi staining in prostate tissue samples indicated that IKKi was more intensively expressed in prostate glands than in prostate stromal fibroblasts. There was no significant difference between IKKi staining intensity in BPH and PC lesions. We also have not revealed correlation between IKKi expression and PC grade. When we used ProSci monoclonal antibody, we have not confirmed our previous finding that IKKi has preferential nuclear localization in epithelial cells in PCs (Fig. 2).

The quality of double staining for IKKi and its potential substrates on paraffin prostate sections was not satisfactory. Thus, we used thin serial sections for the analysis of target protein co-localization with IKKi.

There were no significant changes in the expression of IKKb, IKKa, I-TRAF and TRAF-2 in PC in comparison to BPH (Fig. 1). The immunostaining using antibody against phosphorylated IKB-a (Cell Signaling) was not successful (we could not detect reliable signal after immunostaining). The results of immunostaining with anti-phospho-IKKbeta Ab were published by our group recently (Yemelyanov et al., Oncogene, 2006). Overall analyses of expression have not revealed the significant correlation between IKKi and its potential target localization in PCs.

Task 3. To study the mechanisms of nuclear transport of IKKi and effect of IKKi localization on its function.

Careful analyses of subcellular localization of IKKi in prostate samples and PC cell cultures in vitro using ProSci monoclonal Ab that was the most specific according to Western blot analysis (Fig. 2) have not confirmed our initial finding that IKKi has predominantly nuclear localization. As shown in Fig. 3, both endogenous IKKi expressed in PC3 cells and transgenic IKKi expressed in LNCaP-IKKi cells were mostly localized in the cytoplasm of cells. Due to these findings, we have changed the plan of our research as it was not feasible to study the mechanisms of nuclear localization of IKKi.

As a result, the major focus of our research in FY03 and FY04 was shifted towards biological role of IKKi in prostate cells. We found that IKKi gives growth advantage to PC cells and revealed the

potential involvement of IKKi in the development of hormone refractory stage of PC growth via activation of androgen receptor.

Taking into consideration the newly recognized association of prostate inflammation and prostate cancer that offers one of the greatest opportunities for preventing malignant conversion we continued to study the cross-talk between pro-inflammatory signaling mediated by IKKi and NF-kB and anti-inflammatory signaling mediated by glucocorticoid receptor (GR) in PC cells. We showed that GR functions as a tumor suppressor in prostate cells, and that glucocorticoids and non-steroidal modulators of GR have a strong potential for the treatment of PC patients.

As small inhibitors of IKKi remain to be developed, we searched for other effective strategies of NF-kB blockage in PC cells, and tested growth inhibitory and pro-apoptotic potential of several novel compounds including highly specific IKKβ inhibitors, proteasomal inhibitors as well as dissociated ligands of GR that inhibit NF-kB. We found that proteasomal inhibitors are much more potent than IKK inhibitors in terms of induction of apoptosis in PC cells (Gasparian et al., 2006, abstract # 9).

The detailed findings are described in FY04 report (see above, p. 4 and 5).

Key Research Accomplishments for the entire funding period

Technical achievements:

- We have developed technical protocols for :
  - optimal PC cell stable infection by lentiviruses;
  - optimal regimens of selection of transfected cells;
  - enrichment of cells co-infected with YFP by FACS.
  - Evaluation of growth curves for YFP-infected PC cells using fluorescent plate reader.

- We provided consultations on the lentiviral infection of epithelial cells and post-infection selection for numerous researchers at Northwestern University.

- We have generated several lentiviruses harboring empty vector, IKKi-FLAG, d.n. IKKi-FLAG, and generated stably infected PC3 and LNCaP cell lines with different IKKi status co-infected with YFP-expressing lentivirus.

Research findings:

- We showed that novel inflammation-related upstream IkappaB kinase IKKi is expressed only in highly malignant androgen-independent PC cells lines.

- We found that IKKi is expressed in glandular component of prostate samples including BPH and PC.

- We obtained experimental evidence that IKKi could be involved in the regulation of NF- B activity in PC cells through a positive feedback loop:

  - IKKi is highly inducible in PC cells in NF-kB-dependent fashion. IKKi expression on mRNA and protein levels is increased by NF-kB activators such as IL-1a and TNF-a; and is blocked by NF-kB inhibitors such as proteasome (PS431 and MG132) and IKKbeta (PS1145) inhibitors.

- Transient transfection of PC cell lines with w.t. IKKi resulted in activation of kB.Luciferase reporter, whereas IKKi dominant negative (d.n.) mutant K38A suppressed basal NF-kB activity in those cells.
- Different PC cells stably infected with IKKi-lentivirus had increased basal and inducible NF-kB activity.
- Blockage of IKKi function by transfection of PC3 cells with IKKi d.n. mutant resulted in the decrease of constitutive expression of endogenous κB-responsive genes IκB-α and IL6.

❖ We found that IKKi gives growth advantage to PC cells:

- w.t. IKKi increased LNCaP and PC cell growth in monolayer;
- w.t. IKKi increased tumorigenicity of LNCaP cells assessed in colony forming assay.

❖ Our results suggest that IKKi plays an important role during the transition to hormone refractory stage of PC growth:

- Androgen receptor (AR) was partially translocated to the nucleus and constitutively activated in IKKi-expressing LNCaP cells even in androgen –depleted medium;
- IKKi increased transactivation potential of AR in prostate carcinoma cells;
- LNCaP-IKKi cells became partially androgen-independent and could sustain the androgen ablation.

❖ Taking into consideration the newly recognized association between prostate inflammation and increased risk of PC development, we extended our studies towards cross-talk between pro-inflammatory signaling mediated by IKKi and NF-kB and anti-inflammatory signaling mediated by glucocorticoid receptor (GR) in PC cells.

❖ We showed that glucocorticoids inhibit IKKi expression in PC cells.

❖ We showed that GR functions as a tumor suppressor in prostate cells:

- We found that the expression of glucocorticoid receptor (GR) was dramatically decreased in @ 80% of prostate carcinomas;
- GR inhibited multiple transcriptional factors involved in proliferation and transformation in PC cells, including NF-kB;
- GR decreased expression and inhibited activity of the MAP-kinases (MAPKs) including p38, JNK/SAPK, Mek1/2 and Erk1/2 in PC cells;
- Activated GR signaling resulted in strong inhibition of PC cell growth and normalization of PC cell phenotype assessed by anchorage-independent growth and expression of PC markers.

❖ Compound A, a novel non-steroidal modulator of glucocorticoid receptor that inhibits NF-kB function, inhibited growth and induced apoptosis of highly malignant prostate cancer cells in GR-dependent manner.

Summary of the Reportable Outcomes for the entire funding period:

Manuscripts:

1. Yemelyanov A., Czwornog J., Chebotaev D., and Budunova I. New methods for gene transfer: advantages of lentivirus-mediated gene transduction. R. Lurie Comprehensive Cancer Center Journal, v.X, No1, p. 21-26.

2. Yemelyanov A., Gasparian A., Lindholm P., Dang L., Pierce J., F. Kisseljov, A. Karseladze, Budunova I. Effect of IKK inhibitor PS1145 on NF-kappaB function, proliferation, apoptosis, and invasion activity in prostate carcinoma cells. Oncogene, 2006, 25(3):387-98.

3. Yemelyanov A., Czwornog J., Chebotaev D., Karseladze A., Kulevitch E., Yang X., Budunova I. Tumor suppressor effect of glucocorticoid receptor in prostate. Oncogene, 2007, 26:1885-1896.

4. Nelius T., S. Filleur, A. Yemeyanov, I. Budunova, E. Shroff , Y. Mirochnik, A. Aurora, D. Veliceasa, W. Xiao, Z. Wang, and O.V. Volpert Androgen receptor targets NFkB and TSP1 to suppress prostate tumor growth in vivo. Int J Cancer. 2007, 121(5):999-1008.

5. Yemelyanov A., Czwornog J., Joshi S., Gera L., Budunova I. Compound A, a novel phyto-modulator of steroid hormone receptors, as a candidate for prostate cancer therapy. In revision.

6. Yemelyanov A., Kobzeva V., Budunova I. Role of IKKi in prostate cancer: A link between inflammation and androgen receptor signaling. Manuscript is under preparation.

7. Gasparian A., Yemelyanov A., Chebotaev D., Kisseljov F., and I. Budunova. Targeting NF-κB in prostate carcinoma cells: comparative analysis of proteasome and IKK inhibitors. Manuscript is under preparation.

Abstracts presented at the local and national meetings:

1. Yemelyanov A., Yao Y, and Budunova I. Possible role of IKKi in the constitutive activation of NF-κB in prostate carcinoma cells. Keystone Symposium: NF-kB: biology and pathology. January11-16, 2004, Snowbird, Utah, p. 101.

2. Budunova I. , Yemelyanov A., Gasparian A., Dang L., Pierce J. Effect of IKK-beta specific inhibitor PS1145 on NF-kappaB activity and apoptosis in prostate carcinoma cell lines. Proceedings of AACR *45*, 2004 (abstract # 4572).

3. Yemelyanov, A., Yao, Y.J, and Budunova, I. IKKi is a component of the positive feedback loop involved in the constitutive activation of NF-kB in prostate carcinoma cells. Proceedings of AACR *44:* 852, 2003.

4. Gasparian, A. V., Yao, Y. J., Slaga T.J. and Budunova, I. V. High sensitivity of prostate carcinoma cell lines to NF-kB induction. Proceedings of AACR, *44*: 1451, 2003.

5. Budunova I. , Yemelyanov A., Gasparian A., Dang L., Pierce J. Effect of IKK-beta specific inhibitor PS1145 on NF-kappaB activity and apoptosis in prostate carcinoma cell lines. Proceedings of AACR *45*, 2004 (abstract # 4572).

6. Yemelyanov A., Czwornong J., Chebotaev D., Karseladze A., Yang X., Budunova I. Expression and function of glucocorticoid receptor in prostate carcinomas and PC cells. The Chicago Signal Trunsduction Symposium, May 2005, Chicago, IL.

7. Yemelyanov A., Czwornong J., Chebotaev D., Karseladze A., Yang X., Budunova I. Expression and function of glucocorticoid receptor in prostate carcinomas and PC cells. Keystone Symposium: Hormonal regulation of tumorigenesis. February 20-25, 2005, Monterey, CA, p. 43.

8. Yemelyanov A., Czwornong J., Chebotaev D., Karseladze A., Yang X., Budunova I. Expression and function of glucocorticoid receptor in prostate carcinomas and PC cells. The Chicago Signal Trunsduction Symposium, May 2005, Chicago, IL.

9. Gasparian A., Gasparian N., A. Yemelyanov, D. Chebotaev, F. Kisseljov, and I. Budunova. Targeting NF-κB in prostate carcinoma cells: comparative analysis of proteasome and IKK inhibitors. Keystone Symposium: NF-kB: 20 years on the road from biochemistry to pathology. March 23-28, 2006, Banff, Alberta, Canada, p. 53.

10. Yemelyanov, A. Gasparian, P. Lindholm, L. Dang, F. Kisseljov, A. Karseladze, and I. Budunova. Effects of IKK inhibitor PS1145 on NF-κB function, proliferation, apoptosis and invasion activity in prostate carcinoma cells. 28, 2006, Banff, Alberta, Canada, p. 63.

11. Yemelyanov A., Czwornong J., Chebotaev D., Karseladze A., Yang X., Budunova I. Decreased expression of glucocorticoid receptor in prostate carcinomas and its anti-tumorigenic activity in PC cells *in vitro*. Proceedings of AACR *47*, 2006 (abstract # 5335).

12. Yemelyanov A., Kobzeva V., Budunova I. Role of IKKi in prostate cancer: A link between inflammation and androgen receptor signaling. Proceedings of AACR, 2007 (abstract # LBA-9158).

13. Budunova I. , Yemelyanov A., Gasparian A. Role of IKKs and transcription factor NF-kB in prostate tumorigenesis. P. 23. IMPACT DOD meeting, 2007, Atlanta.

Seminars presented by P.I.

1. Constitutive activation of NF-kB in prostate carcinoma cells: possible role of feedback loop involving IKKi. Department of Urology seminar program, Feinberg School of Medicine, Northwestern University, Chicago, September, 2003.

2. Effect of NF-kB inhibitor PS1145 and glucocorticoids on prostate carcinoma cells.
Prostate SPORE, R. Lurie Cancer Center, Northwestern University, Chicago, IL, September 2004.

3. Targeting NF-kB transcription factor and IKK kinases in prostate carcinoma cells. The University of Auckland, School of Medicine-Auckland Cancer Society Research Center, Auckland , New Zealand , November 2004

4. Targeting the transcription factor NF-kB and up-stream kinases for intervention of prostate and skin cancer. Ludwig Institute for Cancer Research and Royal Melbourne Hospital, Melbourne, Australia, November 2004.

5. Constitutively active NF-κB transcription factor and IKKb kinase in human prostate carcinoma cells as

a possible targets for intervention. Epithelial group seminar series. R.Lurie Cancer Center. Northwestern University. December, 2004.

6. Invited oral presentation. Expression and function of glucocorticoid receptor in prostate carcinomas and PC cells. Keystone Symposium:Hormonal regulation of tumorigenesis. February 2005, Monterey, CA, p. 43.

7. Compound A, a novel phyto-modulator of steroid hormone receptors, as a candidate for prostate cancer therapy. Tumor cell biology seminars. R. Lurie Cancer Center. NU. April, 2007.

8. Non-steroidal modulators of steroid hormone receptors as candidates for prostate cancer therapy. Children's Memorial Research Center, Chicago. May , 2007

Conclusions the entire funding period:

We found that novel inflammation-related upstream IkappaB kinase IKKi is expressed only in highly malignant androgen-independent PC cells lines. IKKi is also expressed in epithelial cells in benign and malignant prostate lesions. Our data provide experimental evidence that IKKi could be involved in the regulation of activity of major anti-apoptotic factor NF-κB in PC cells through a positive feedback loop. Our results also suggest that IKKi may play an important role during the transition to hormone refractory stage of PC growth via its positive effect on the nuclear translocation and activity of androgen receptor in PC cells. Taking into consideration the newly recognized association between prostate inflammation and increased risk of PC development, we extended our studies towards cross-talk between pro-inflammatory signaling mediated by IKKi and NF-kB and anti-inflammatory signaling mediated by glucocorticoid receptor (GR) in PC cells. We showed that GR functions as a tumor suppressor in prostate cells, and that inhibition of transcription factors involved in proliferation and transformation in PC cells, including NF-kB, is the major molecular mechanism of GR anti-tumor activity. Finally, we discovered that Compound A, a novel non-steroidal modulator of glucocorticoid receptor that inhibits NF-kB function, inhibited growth and induced apoptosis of highly malignant prostate cancer cells in GR-dependent manner.

List of personnel receiving pay from the research effort for the entire funding period:

1. Irina Budunova, M.D., Ph.D., P.I.
2. Alexander Yemelyanov, M.D., Ph.D., program investigator
3. Vera Kobzeva, Ph.D., program investigator
4. Chebotaev Dmitry, Ph.D., program investigator
5. Czwornog Jennifer, Research Assistant

Collaborations for the entire funding period:

In 2003 P.I. became a member of R. Lurie Comprehensive Cancer Center/ Northwestern University Prostate SPORE. In frames of prostate SPORE P.I. is collaborating with Dr. O. Volpert , an Associate professor at the Department of Urology at Northwestern University to study the effect of NF-kB blockage on the function of androgen receptor. This collaboration is reflected in the publication Nelius et al., 2007.

P.I. also started the collaboration with the pathologist Dr. X. Yang, a Professor at the Department of Pathology at Northwestern to study the expression of IKKs and steroid hormone receptors in PCs. The results of collaboration are reflected in the manuscript Yemelyanov et al., 2007.

P.I. continues collaboration with Dr. A. Karseladze, Chair of the Department of Molecular Pathology at N. Blokhin Cancer Research Center (Moscow, Russia) to study the expression of NF-kB and IKKs in PCs. The results of collaboration are reflected in two manuscripts Yemelyanov et al., 2006, Yemelyanov et al., 2007.

Further, P.I. initiated collaboration with Dr. P. Lindholm, an Associate Professor at the Department of Pathology at Northwestern University to study the effect of IKK inhibition on PC cell invasion. The results of this collaboration are included in the manuscript published by Yemelyanov et al.,2006.

During funding period (06.2003-06.2007) P.I., Dr. Irina Budunova has expanded her work towards the search for most effective strategies of NF-kB blockage in PC cells. Her group tested several novel compounds including highly specific IKKβ inhibitor PS1145, proteasomal inhibitor PS341/Velcade (both in collaboration with Millenium Pharmaceuticals Inc. , Cambridge, MA), as well as dissociated ligands of glucocorticoid receptor that inhibit NF-kB via stimulation of negative protein/protein interaction between activated GR and p65, including AL438 (in collaboration with Ligand Pharmaceuticals, San Diego, CA).

Promotions:

The key researcher on this grant, Dr. A. Yemelyanov was promoted to the position of Assistant Professor at the Department of Dermatology, School of Medicine at Northwestern University (Chicago, IL).

Additional funding obtained/applied based on the work supported by award:

In 2004 P.I. initiated studies to evaluate the combined effect of glucocorticoids and IKK inhibitors on PC cell growth. This research "Combinational targeting of NF-kB transcription factor as a novel strategy for apoptosis induction and prostate carcinoma treatment" is supported by developmental project award (to Budunova IV) from Northwestern University Prostate SPORE 5 P50 CA090386-04 (P.I. C. Lee).

Looking for the combinational therapeutic approaches to block NF-kB in prostate P.I. became interested in steroidal and non-steroidal ligands of glucocorticoid receptor that potently inhibit NF-kB through negative interaction on protein-protein level. We also showed that glucocorticoids inhibit the expression of IKKi on PC cells. In 2007 P.I. submitted grants "Role of glucocorticoid receptor in prostate tumorigenesis: from experimental studies to clinical applications" and "Compound A, a novel phyto-modulator of steroid hormone receptors, as a candidate for prostate cancer therapy" to NIH and DOD. In 2007 proposals have not been funded; grants will be resubmitted after revision to NIH and DOD prostate program in 2008.

Training of postdoctoral fellows:

1. **Dr. Dmitry Chebotaev**, 2003- 2006 , Department of Dermatology, NU, Chicago, IL.
   Currently – Senior Researcher at the Applied Biosystems, Moscow, Russia
2. **Dr. Vera Kobzeva**, 2005-2006, Department of Dermatology, NU, Chicago, IL.
   Currently – Program Investigator, Institute of Carcinogenesis, Blokhin Cancer Center, Moscow, Russia.

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Appendices:

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2. T. Nelius, S. Filleur, A. Yemeyanov, I. Budunova, E. Shroff , Y. Mirochnik, A. Aurora, D. Veliceasa, W. Xiao, Z. Wang, and O.V. Volpert Androgen receptor targets NFkB and TSP1 to suppress prostate tumor growth in vivo. Int J Cancer. 2007, 121(5):999-1008.
3. Yemelyanov A., Kobzeva V., Budunova I. Role of IKKi in prostate cancer: A link between inflammation and androgen receptor signaling. Proceedings of AACR, 2007 (abstract # LBA-9158).
4. Budunova I. , Yemelyanov A., Gasparian A. Role of IKKs and transcription factor NF-kB in prostate tumorigenesis. P. IMPACT DOD meeting, 2007, Atlanta.

Supplemental figures:

Figure 1. Expression and localization of IKKs and their substrates in benign (BPH) and malignant (PC) lesions.

Figure 2. Comparison of IKKi antibodies : application for Western blotting and immunostaining.

Figure 3. Preferential cytoplasm localization of IKKi

Figure 4. IKKi is involved in constitutive activation of NF-kB in PC cells through a positive feedback loop.

Figure 5. IKKi overexpression results in the increased proliferation and tumorigenicity of PC cells.

Figure 6. Effect of IKKi on NF-kB status in PC cells.

Figure 7. IKKi overexpression results in increased nuclear localization of AR

Figure 8. IKKi overexpression results in increased AR function and PC cell resistance to androgen ablation.

Figure 9. Effect of CpdA on GR function in PC cells.

Figure 10. CpdA inhibits growth of PC cells.

Figure 11. CpdA induces apoptosis of PC cells.

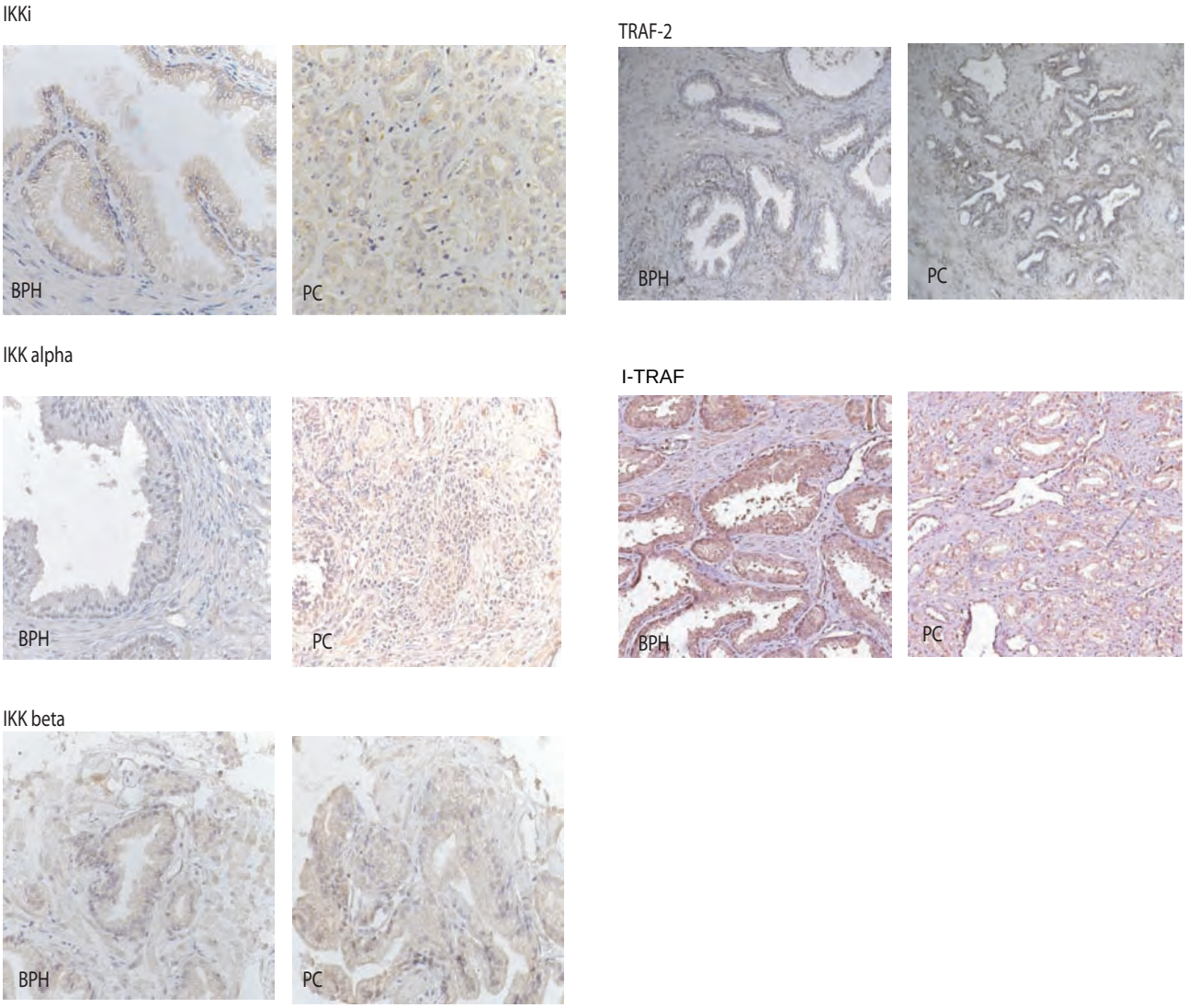


Figure 1. Expression of IKKs and IKK substrates in benign (BPH) and malignant (prostate cancer) lesions.
Formalin-fixed, paraffin-embedded sections of benign (benign prostate hyperplasia, BPH) and malignant (PC, prostate carcinoma) lesions were used for immunostaining with anti-IKKi (ProSci, Poway, CA) , anti-I-TRAF, anti-IKKβ, anti-IKKα and anti TRAF-2 Abs (all from Santa Cruz Technology, Santa Cruz, CA).

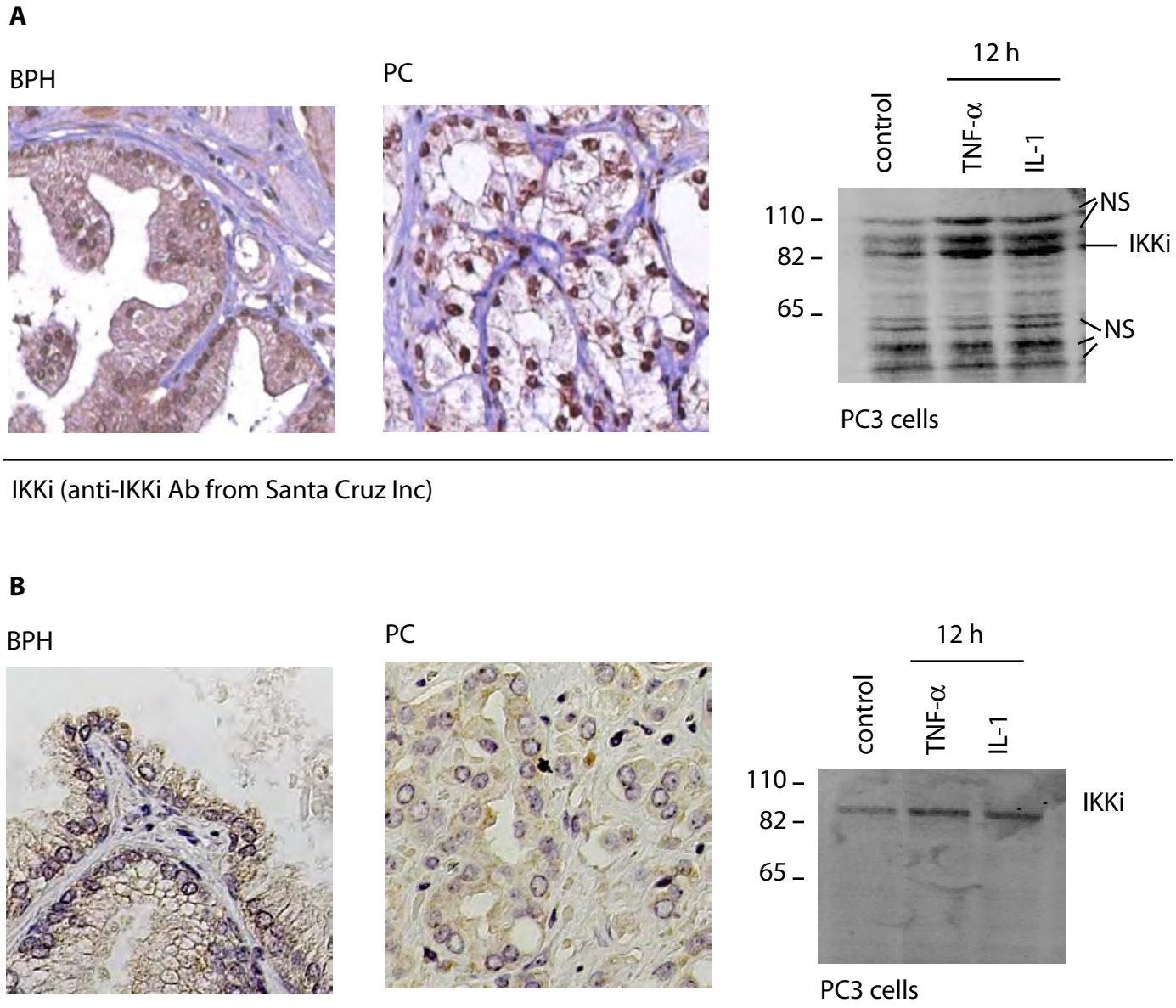


Figure 2. Comparison of IKKi antibodies: application for Western blotting and immunostaining.
Formalin-fixed, paraffin-embedded sections of benign (benign prostate hyperplasia , BPH) and malignant (PC, prostate carcinoma) lesions were used for immunostaining with two different anti-IKKi antibodies: **A** - from Santa Cruz Biotech (Santa Cruz, CA) and **B** - from ProSci laboratories (Poway, CA).
PC3 cells that expressed endogenous IKKi , were treated with NF-κB inducers TNF-α (10 nM) and IL-1 (1 M), whole-cell proteins were extracted as described in Yemelyanov et al., 2006.
Note: only anti-IKKi polyclonal Ab from Santa Cruz Inc that gave multiple bands on Western blots detected significant amount of IKKi in the nuclei of prostate cells in PC. NS - non-specific protein bands.

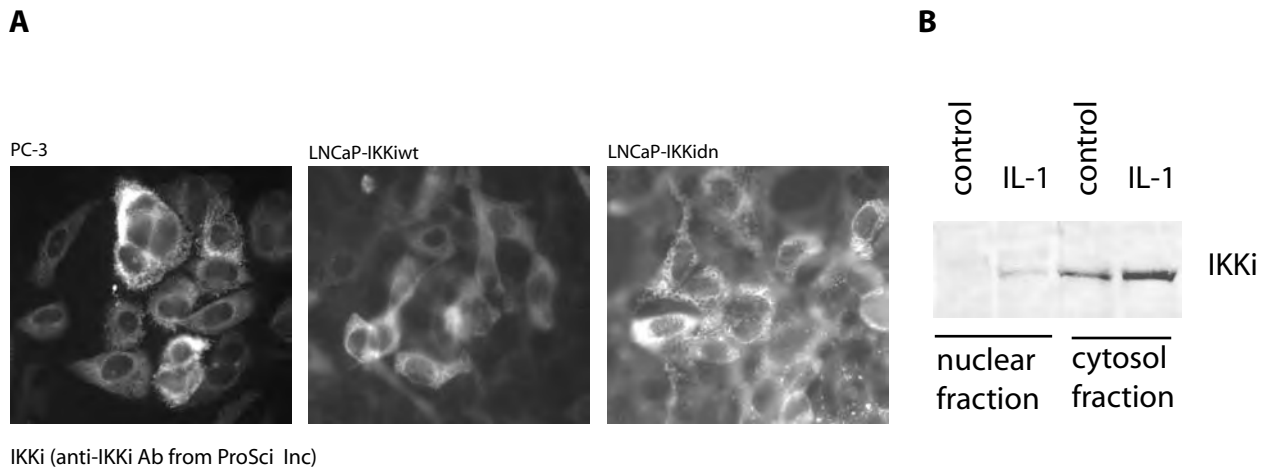


Figure 3. Preferential cytoplasmic localization of IKKi. A - Cell were fixed with formalin, permebealized with methanol : acetone mix (1:1), blocked with 10% goat serum and stained with anti-IKKi antibodies (ProSci, Poway, CA) and secondary Abs conjugated with Cy3 (Jackson Immunolabs, (West Grove, PA). **B** - Nuclear and cytosol protein fractions were isolated from LNCaP cells expressing endogenous IKKi w.t. and analyzed by Western blotting using anti-IKKi Abs (ProSci, Poway, CA). **Note**: Endogenous and lentivirus-expressed exogenous IKKi were localized at most to the cell cytoplasm. Stimulation of the cells with IL-1 induced very little IKKi nuclear translocation.

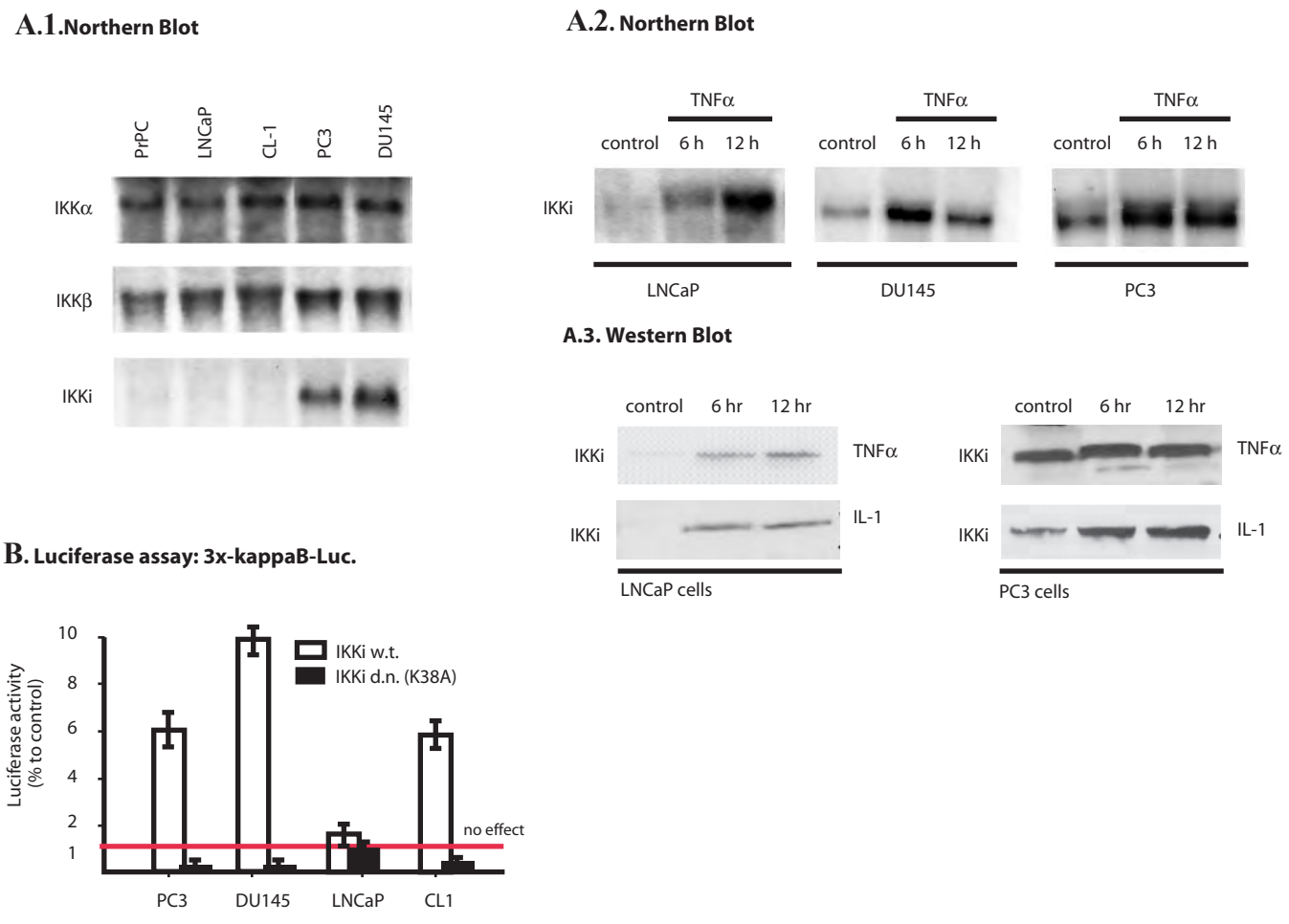


Figure 4. IKKi is involved in constitutive activation of NF-κB in prostate carcinoma cells through a positive feedback loop. A1. Expression of IKKα, IKKβ and IKKi in PC cell lines. Northern blot analysis of IKKα, IKKβ and IKKi expression in prostate cells. **Note:** IKKi is expressed only in highly malignant PC3 and DU145 cells. **A2** and **A3**. Induction of IKKi in prostate cells by cytokines. Prostate cells were treated with TNFα (10 ng/ml), and IL-1 (1 μg/ml), RNA and whole cell proteins were isolated and used for Northern and Western blot analyses to evaluate IKKi expression. **B**. Effect of IKKi w.t. and d.n. IKKi mutant on NF-κB activity in PC cells. Prostate cells were cotransfected with 5x-κB-Luciferase reporter (FL), Renila luciferase (RL) under minimal promoter, and either IKKi w.t. or IKKi d.n mutant (kindly provided by Dr. T. Maniatis, Harvard University, Harvard, MA). Luciferase activity was measured by dual luciferase assay. FL activity was normalized against RL activity to equilize for transfection efficacy. **Note:** IKKi w.t. induced NF-κB activity and IKKi d.n. mutant K38A inhibited NF-κB activity in PC cells.

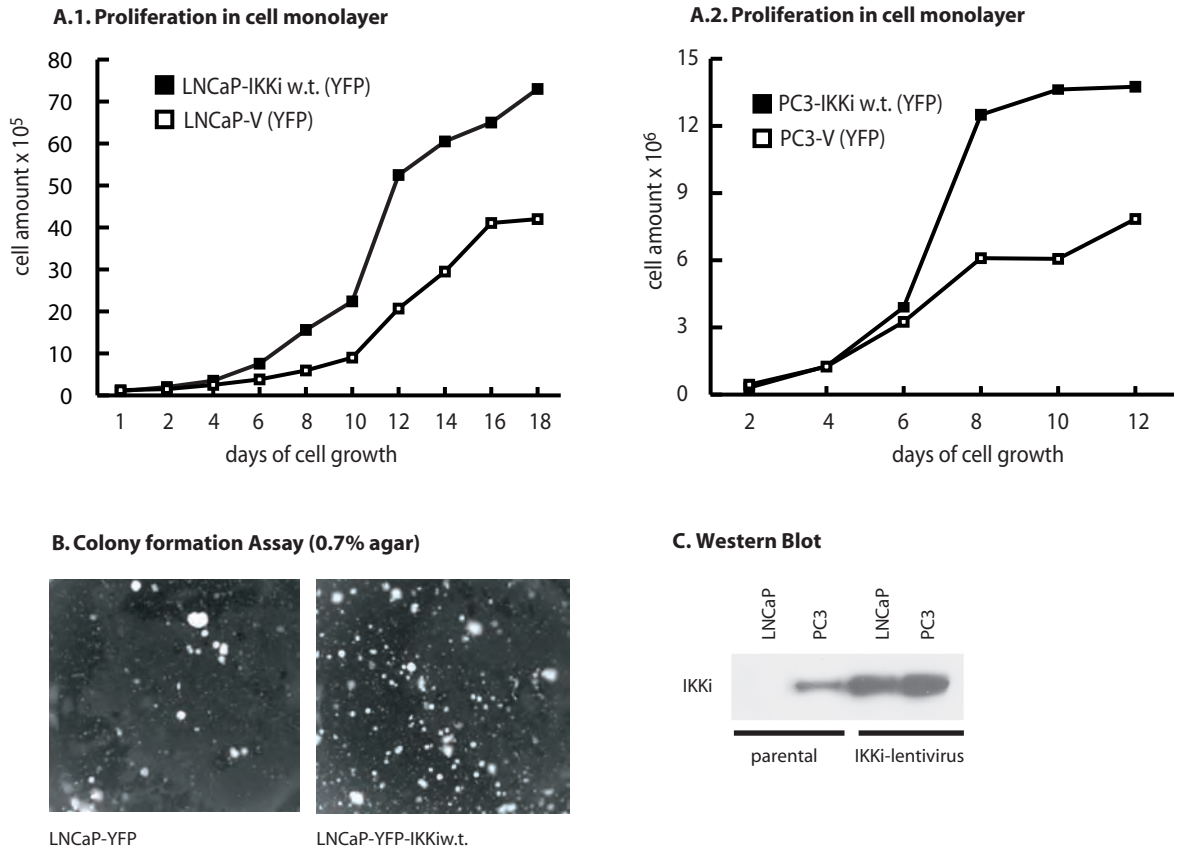


Figure 5. IKKi overexpression results in the increased proliferation and tumorigenicity of PC cells. Generation of IKKi w.t. cells. PC3 and LNCaP cells were stably infected with lentivirus expressing human IKKi cDNA (kindly provided by Dr. T. Maniatis, Harvard University, Harvard, MA). For easier tracking, the additional cell lines were co-infected with IKKi and yellow fluorescent protein (YFP) lentiviruses. Control cell lines were infected with either the empty lentivirus (LNCaP-V, PC3-V) or the lentivirus expressing YFP (LNCaP-YFP; PC3-YFP). **A.** Effect of IKKi on proliferation of prostate cells. Number of IKKi- and empty virus infected was calculated by hemocytometer. **B.** Effect of IKKi on anchorage independent growth of LNCaP-YFP cells. LNCaP-IKKi-YFP and LNCaP-V-YFP cells were grown in 0.7% soft agar for 3 weeks. Images were obtained by AxioVert 40 CFL inverted microscope equipped with a fluorescent digital camera (Zeiss). **C**. Western blot analysis of IKKi expression in transfected prostate cell lines.

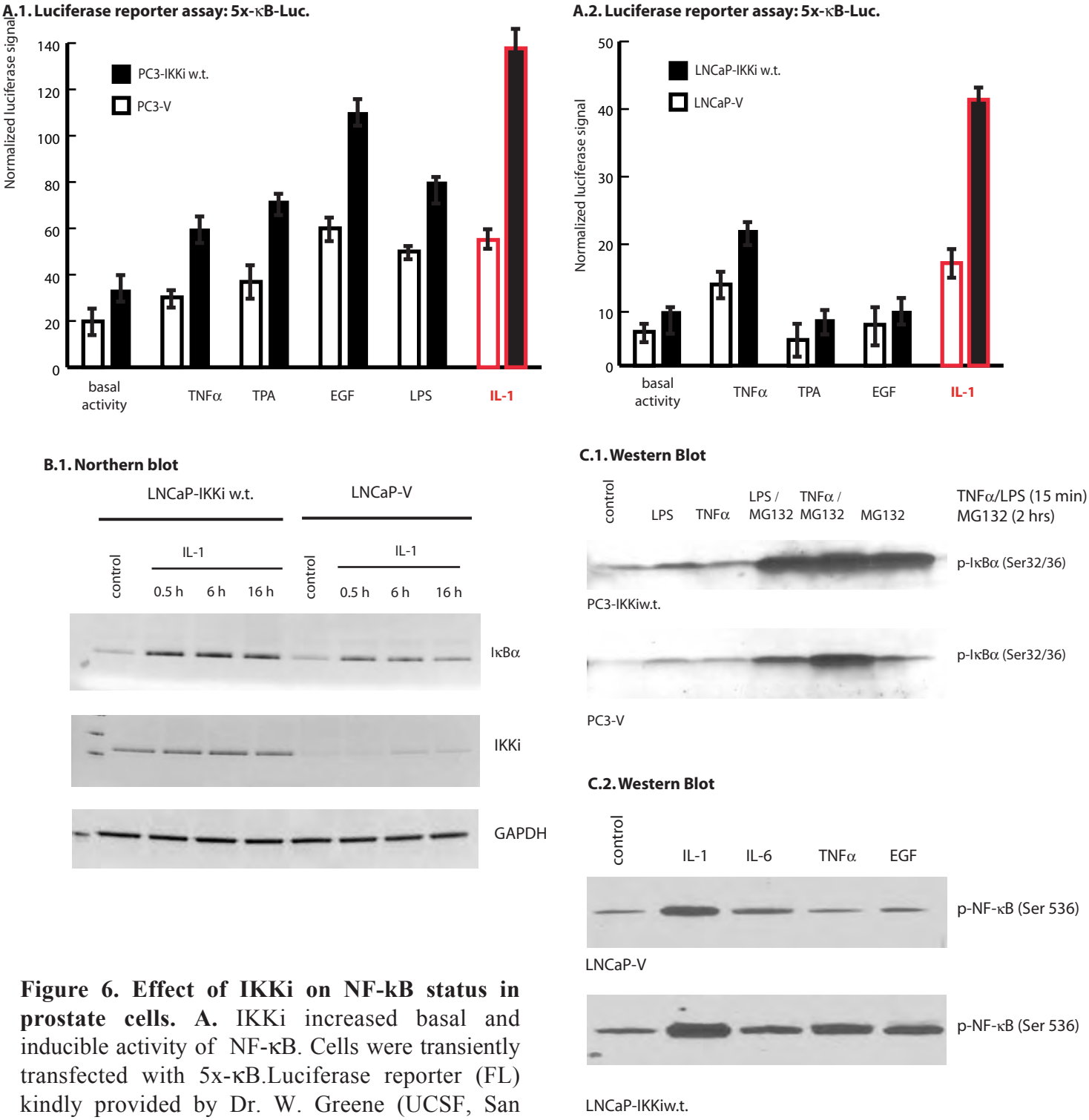


Figure 6. Effect of IKKi on NF-kB status in prostate cells. A. IKKi increased basal and inducible activity of NF-κB. Cells were transiently transfected with 5x-κB.Luciferase reporter (FL) kindly provided by Dr. W. Greene (UCSF, San Francisco, CA) and with Renilla Luciferase (RL) under minimal promoter. Cells were treated with TNFa (10 ng/ml), IL-1 (1 μg/ml) and EGF (100 ng/ml), LPS (1 μg/ml) and TPA (10 μg/ml) for 24 hrs, and Luciferase activity was measured by dual Luciferase assay. FL activity was normalized against RL activity to equalize for transfection efficacy. Note: in both PC cell lines IKKi most significantly increased NF-κB induction by IL-1. **B.** IKKi increased expression of endogenous κB-dependent genes. Cells were treated with IL-1 (1 μg/ml), total RNA was isolated by TRI reagent (Molecular Research Center, Inc., Cincinnati, OH) and subjected to Northern blotting. Membranes were probed for NF-κB regulated genes: IκBa and IKKi, and for GAPDH as a control for RNA loading. **Note:** in both PC cell lines IKKi significantly increased the expression of κB-dependent genes. **C.** IKKi significantly increased phosphorylation of IκBa (Ser32/Ser36) and p65 (Ser536). To better assess phosphorylation, cells were pre-treated with proteasomal inhibitor MG132 (x2 hr), and treated with NF-kB inducers. Whole cell protein extracts were used for Western blot analysis with anti-phospho-Ser32/Ser36 IκBa and anti-phospho-Ser536 p65 Abs (Cell Signaling, Beverly, MA).

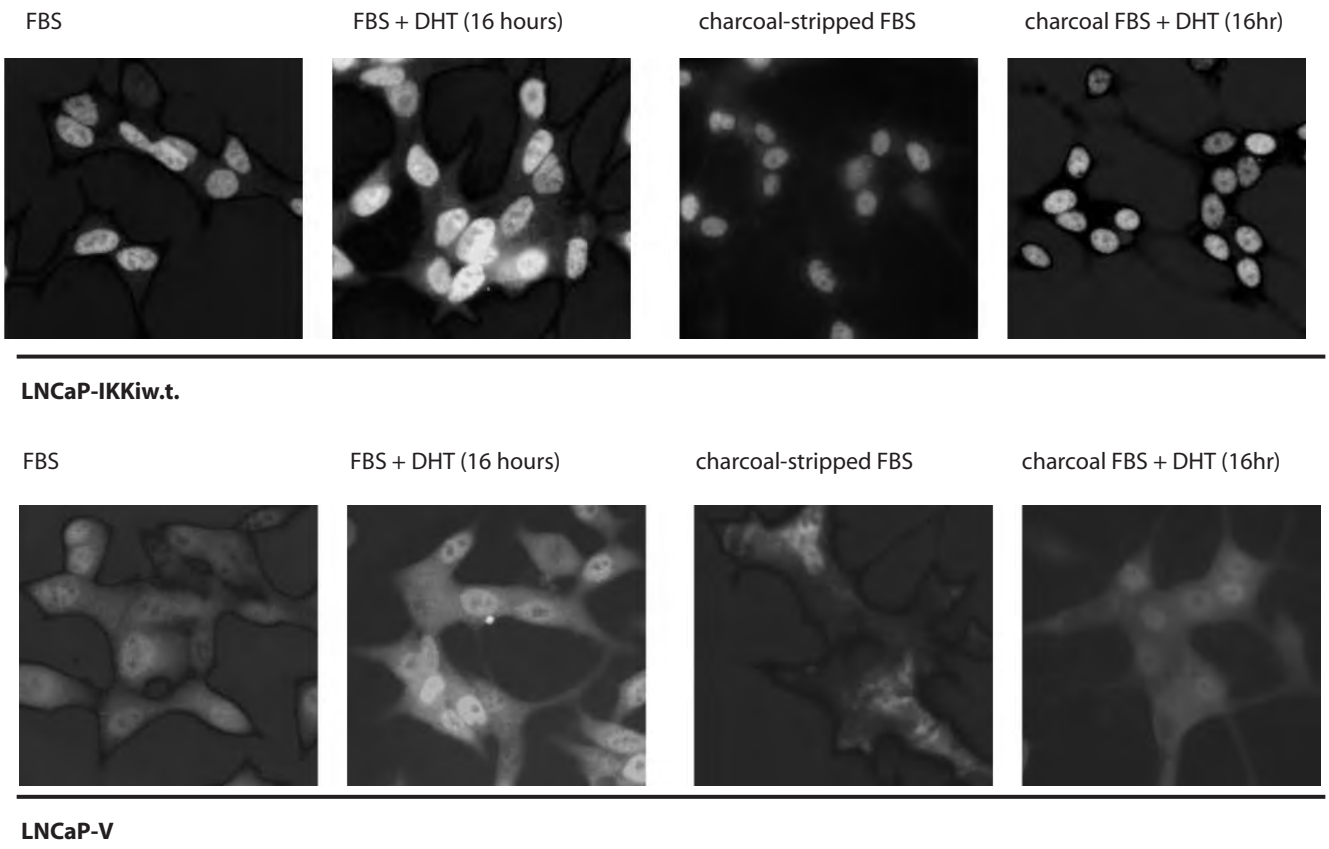


Figure 7. IKKi induces AR nuclear localization. IKKi increased nuclear localization of AR induced by DHT. LNCaP-V and LNCaP-IKKw.t.i cells grown in the medium with normal and charcoal-stripped serum were treated with DHT (10^{-7} x 24 hrs), fixed and used for immunofluorescence with anti-AR Ab (Santa Cruz Biotechnology) followed by Cy-3-conjugated anti-mouse secondary Ab conjugated with Cy3 .

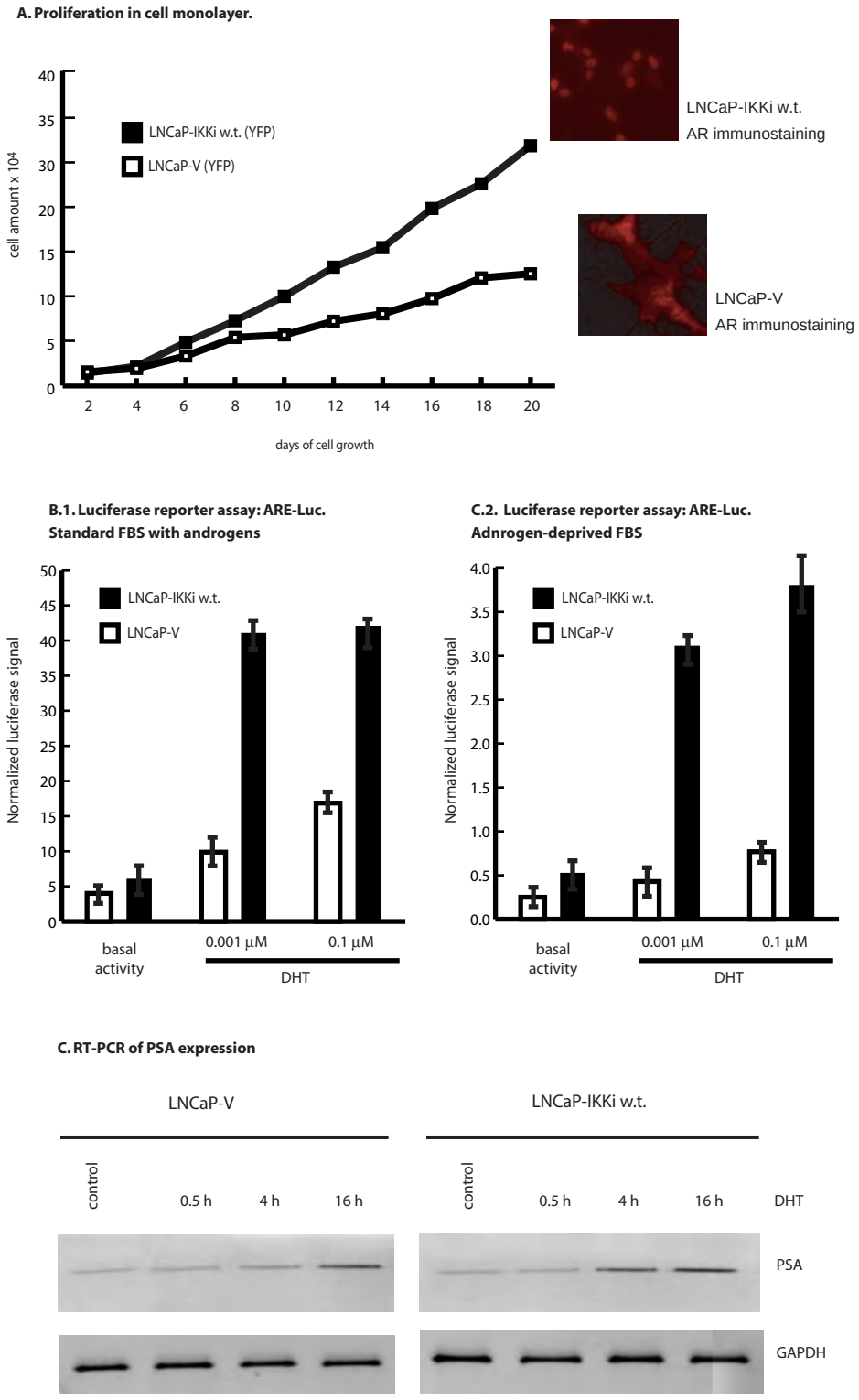
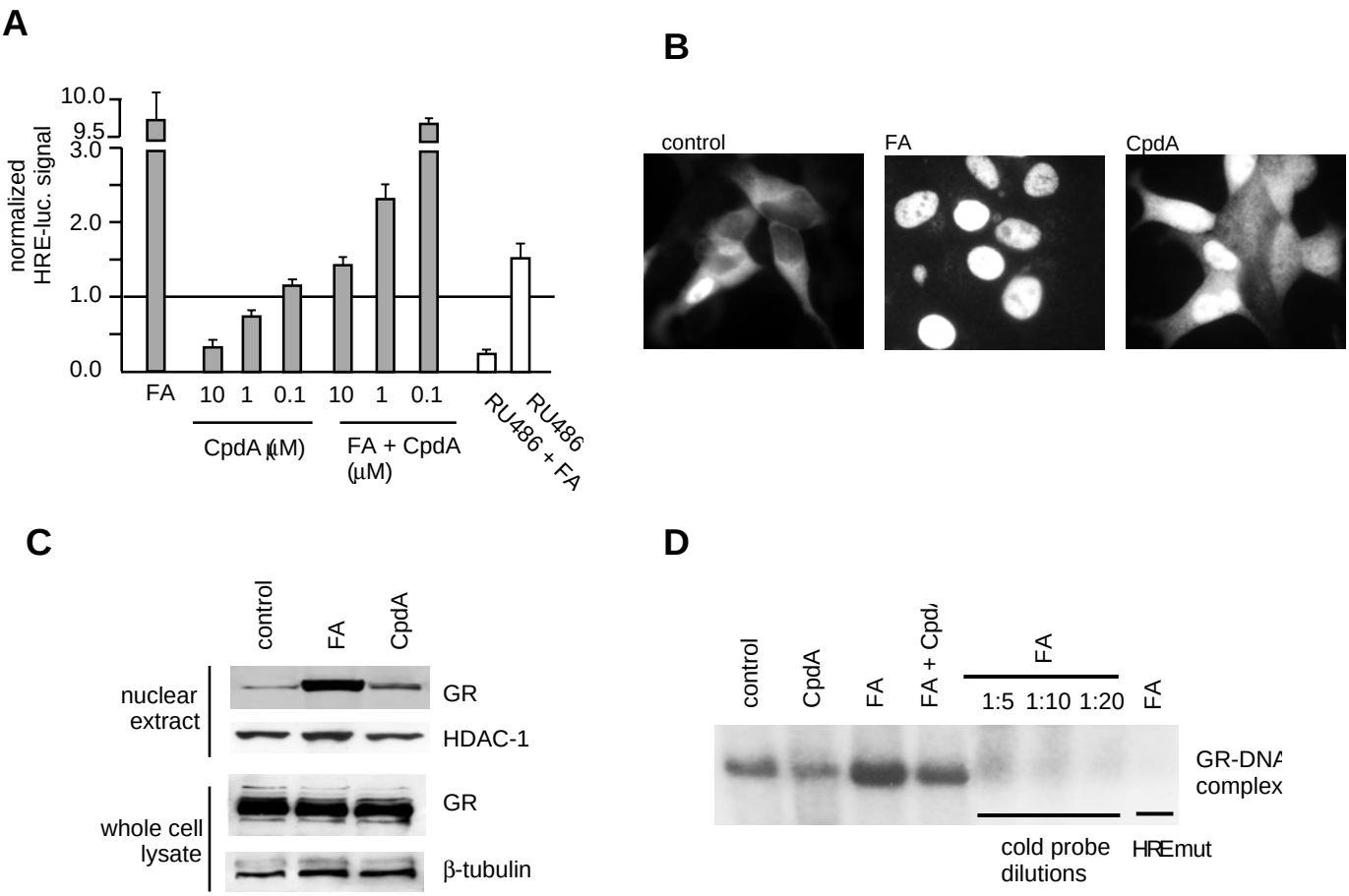


Figure 8. IKKi increased AR function and PC cell resistance to androgen ablation (B-D). A. Increased resistance of LNCaP-IKKi cells to androgen ablation. LNCaP-IKKi and LNCaP-V cells were grown in the medium with normal and charcoal-stripped serum. Cell number was calculated by hemocytometer. **B**. Increased basal and DHT-induced activity of AR in LNCaP-IKKi cells. LNCaP-V and LNCaP-AR cells were transfected with MMTV.Luciferase reporter (FL) (ARE.Luc) and with Renilla Luciferase (RL) under minimal promoter. Cells were treated with DHT (10^{-7} x 36 hrs) in the medium with normal or charcoal-stripped serum. Luciferase activity was measured by dual Luciferase assay. FL activity was normalized against RL activity to equalize for transfection efficacy. **C**. Increased PSA expression in LNCaP-IKKiw.t. cells. LNCaP-V and LNCaP-IKKiw.t. cells grown in the medium with normal and charcoal-stripped serum were treated with DHT (10^{-7} 0.5-16 hrs). Total RNA was isolated and used for RT-PCR analysis of PSA expression.



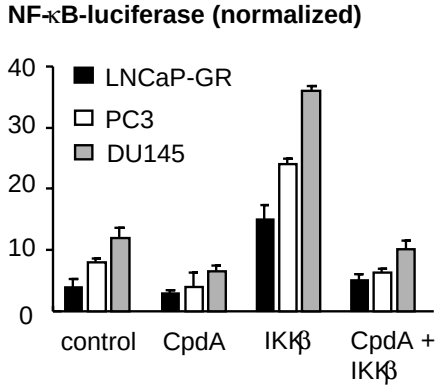


Figure 9. Effect of CpdA on GR function in PC cells.

B, C. CpdA activated GR nuclear translocation. Cells were treated with CpdA, glucocorticoid FA, and anti-glucocorticoid RU486; **B** – immunofluorescence; **C** - Western blotting, nuclear proteins. HDAC-1 and b-tubulin were used as protein loading controls.

D. CpdA decreased GR-DNA binding. EMSA, nuclear proteins. LNCaP-GR cells were treated with 0.01% DMSO (control), Dex (10^{-7} M) and CpdA (10^{-5} M) for 4 hrs.

A, E. CpdA effect on GR function. PC cells were transiently transfected with TAT.Luc (**A**) or κB.Luc (**E**), and control Renilla Luciferase reporters, treated with Dex (10^{-6} M) and CpdA (10^{-5} M) for 24 hrs. For activation of NF-κB cells were co-transfected with CMV.IKKβ plasmid (in **E**). Reporter activity was assessed by dual Luciferase assay, and presented as factor of change.

Note: CpdA induces GR nuclear translocation; inhibits DNA binding and GR transactivation, but induces GR transrepression in Luciferase assay.

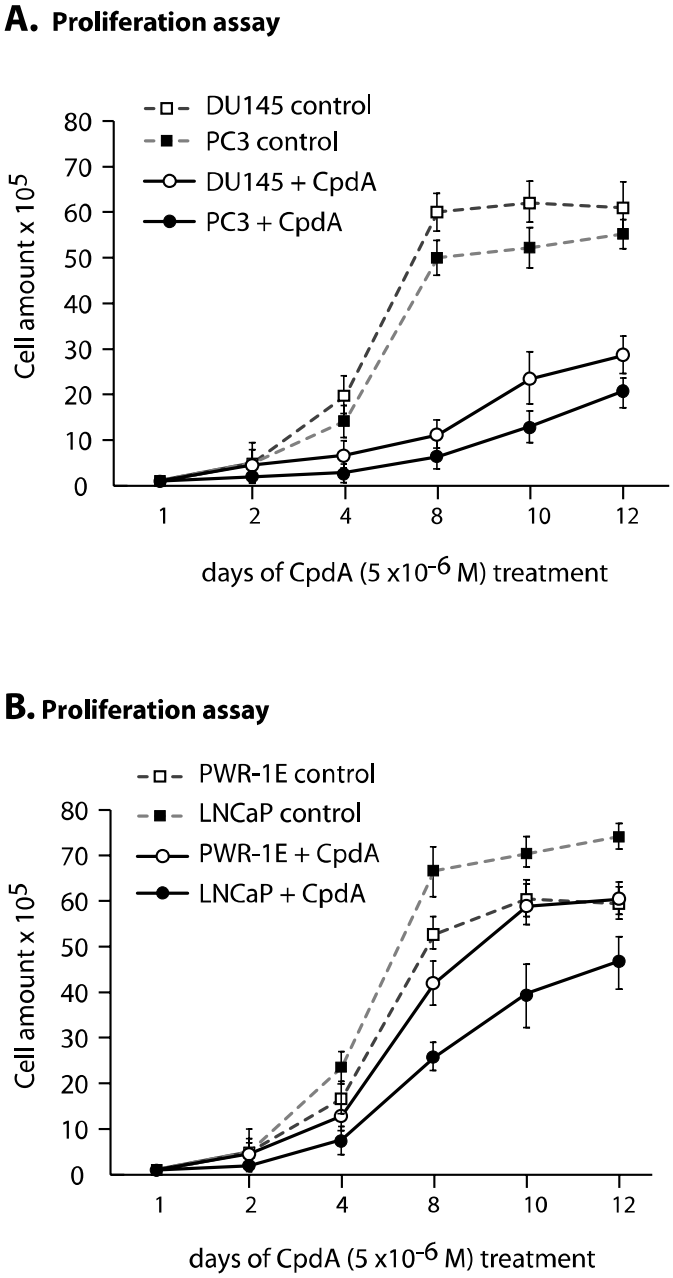


Figure 10. Highly malignant PC cells are sensitive to the growth inhibitory effect of CpdA. A, B. Non-transformed prostate cells PWR-1E and PC cells LNCaP, DU145 and PC3 were treated with 0.01% DMSO (control) or CpdA ($5x10^{-6}$ M) for 1-12 days. Cell number/well was determined by counting, and the absolute number of cells/well is presented as mean +/- S.D for each experimental group (three wells/group).
Note: high sensitivity of androgen-independent PC3 and DU145 cells to CpdA (Fig. 5A).

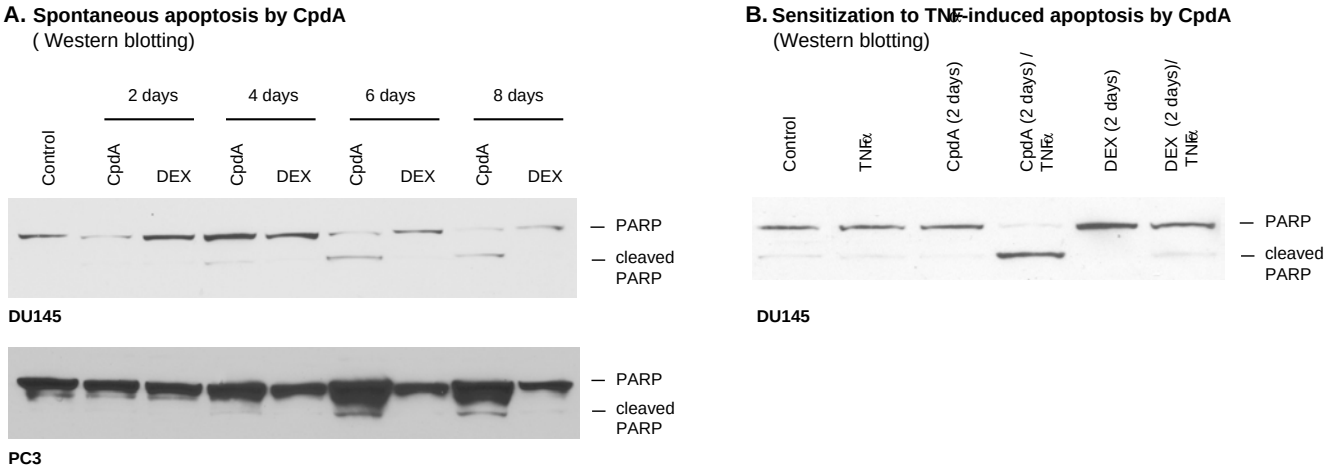


Figure 11. CpdA induces apoptosis in prostate cells. A. PC3 and DU145 cells were treated for 1-8 days with DMSO (control), CpdA ($2x10^{-6}$ M) and Dexamethasone (10^{-6} M). Nuclear cell extracts were analyzed for PARP cleavage by Western blotting using anti-PARP antibody (Cell Signaling, Danvers, MA). B. To study prostate cell sensitization to apoptosis by CpdA we used TNFα (10 ng/ml x 16 hrs).
Note: CpdA induced apoptosis after 6-8 day treatment and sensitized cells to TNFα-induced apoptosis after 2 day treatment.

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ORIGINAL ARTICLE

Tumor suppressor activity of glucocorticoid receptor in the prostate

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Glucocorticoids are extensively used in combination chemotherapy of advanced prostate cancer (PC). Little is known, however, about the status of the glucocorticoid receptor (GR) in PC. We evaluated over 200 prostate samples and determined that GR expression was strongly decreased or absent in 70–85% of PC. Similar to PC tumors, some PC cell lines, including LNCaP, also lack GR. To understand the role of GR, we reconstituted its expression in LNCaP cells using lentiviral approach. Treatment of LNCaP-GR cells with the glucocorticoids strongly inhibited proliferation in the monolayer cultures and blocked anchorage-independent growth. This was accompanied by upregulation of p21 and p27, downregulation of cyclin D1 expression and c-Myc phosphorylation. Importantly, the activation of GR resulted in normalized expression of PC markers hepsin, AMACR, and maspin. On the signaling level, GR decreased expression and inhibited activity of the MAP-kinases (MAPKs) including p38, JNK/SAPK, Mek1/2 and Erk1/2. We also found that activation of GR inhibited activity of numerous transcription factors (TF) including AP-1, SRF, NF-κB, p53, ATF-2, CEBPα, Ets-1, Elk-1, STAT1 and others, many of which are regulated via MAPK cascade. The structural analysis of hepsin and AMACR promoters provided the mechanistic rationale for PC marker downregulation by glucocorticoids via inhibition of specific TFs. Our data suggest that GR functions as a tumor suppressor in prostate, and inhibits multiple signaling pathways and transcriptional factors involved in proliferation and transformation.
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Keywords: prostate carcinoma; PIN; glucocorticoid receptor; PC marker; transcription factor; MAPKs

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Introduction

Glucocorticoid hormones regulate proliferative, inflammatory and immune responses. For years, glucocorticoids have been extensively used for the treatment of hormone refractory prostate cancer (HRPC), and the combination of paclitaxel and dexamethasone remains a standard treatment for HRPC patients in the US and other countries (reviewed by Fakih *et al*., 2002). Glucocorticoids have also been used as the 'standard' therapy arm in several randomized phase II–III clinical trials for the combination therapy of HRPC (Fakih *et al*., 2002; Koutsilieris *et al*., 2002).

The cellular response to glucocorticoids is mediated through a highly specific glucocorticoid receptor (GR). In the absence of glucocorticoids, GR is sequestered in the cytoplasm by chaperone proteins. Following ligand binding, the GR dissociates from the chaperones and forms homodimers, which enter the nucleus. There are two major mechanisms of gene regulation by GR (De Bosscher *et al*., 2003; Necela and Cidlowski, 2004). The direct positive transcriptional regulation (transactivation) occurs via binding of the GR homodimer to palindromic promoter DNA sequences called glucocorticoid-response elements. The indirect regulation is mediated via crosstalk with other transcription factors (TFs), including activator protein 1 (AP-1), nuclear factor kappa-B (NF-κB), signal transducer and activator of transcription (STAT)-5, mothers against DPP homolog 3 (SMAD3), etc. (De Bosscher *et al*., 2003; Necela and Cidlowski, 2004). Most of such GR–TF interactions repress the activity of partner TFs and their target genes (transrepression). Recently, the additional mechanism of indirect gene regulation by GR has been discovered where GR blocks mitogen-activated protein kinases (MAPKs) (Kassel *et al*., 2001; Bruna *et al*., 2003). Indirect, DNA-independent mechanisms of GR gene regulation appear to be critical for the anti-inflammatory effects (Schacke *et al*., 2002), whereas their role in the growth inhibition by glucocorticoids has never been addressed.

Although the clinical effect of glucocorticoids in HRCP patients is well known, the objective responses have been found only in 20–25% of patients (Fakih *et al*., 2002). The limited effect of glucocorticoids in prostate carcinoma (PC) patients implies the changes in

GR expression, function and/or availability of GR targets in PC cells. Indeed, we and others showed that different types of tumor cells lose their sensitivity to growth inhibition and apoptosis by glucocorticoids either because of the loss of GR expression or because of the abnormal GR function (Ray, 1996; Budunova *et al*., 1997; Greenstein *et al*., 2002). These observations suggest that intact GR signaling is crucial for the growth control of lymphoid and epithelial cells and that in some tissues GR may act as a tumor suppressor.

Despite the use of glucocorticoids in the standard combinational therapies of PC patients, the information regarding GR expression in PCs is surprisingly limited and conflicting (Mohler *et al*., 1996; Nishimura *et al*., 2001). To our knowledge, GR expression in early prostate lesions such as intraepithelial neoplasia (PIN) has never been evaluated. Furthermore, GR function in the prostate cells and its role in PC have never been studied, even though the growth inhibitory effect of glucocorticoids in GR-positive human and rat prostate cells has been reported (Nishimura *et al*., 2001). These previous studies chiefly attribute growth inhibitory effect of glucocorticoids to the inhibition of NF-κB TF (Nishimura *et al*., 2001).

Here, we for the first time present the comprehensive analysis of GR expression changes in the course of prostate tumorigenesis, and determine the effect of activated GR signaling on proliferation and the maintenance of transformed phenotype by PC cells.

Results

The expression of GR is decreased in HGPIN and PCs

We analysed GR expression in prostatic tissue specimens retrieved from the two independent repositories. Overall, we evaluated GR expression in 35 high-grade prostatic intraepithelial neoplasia (HGPIN) lesions, 116 PC samples (sum Gleason grades 6–10) and in 67 benign prostatic hyperplasia (BPH) samples.

The results of GR immunostaining appeared to be very similar between the cohorts (Figure 1 and Table 1). More than 80% of BPH samples showed high-intensity GR staining with nuclear localization in the epithelial cells (Figure 1a). Strong GR staining was localized to the nuclei in most of the glands in apparently normal prostatic tissues (Figure 1b). The nuclear localization strongly suggests that GR is constitutively active in both normal and hyperplastic prostate glands. In contrast, GR levels were low or below detection limit in 70–85% of PCs. There was no association between GR expression levels and Gleason grade of PCs in both cohorts. The lack of dynamics in GR expression during PC progression suggests that it is lost early in prostate tumorigenesis. Indeed, we found that GR expression was significantly decreased in 37% and partially decreased in 40–50% of HGPIN lesions compared to the morphologically normal prostate and BPH glands. However, the average number of GR-positive cells in HGPIN epithelium was almost twofold higher than in PC samples (Figure 1c). Although the analysis of prostate stroma was beyond the scope of this study, we noted that GR was present at high level in the nuclei of stromal cells (Figure 1d). Overall the immunostaining showed the decrease in GR expression to be an early event in prostate tumorigenesis, and suggested that GR may be important to control the growth of prostate cells.

Table 1 GR expression is strongly decreased in prostate carcinomas.

Tissue samples	*Patient cohorts*	*Number of samples*	*GR intensity score**		
			+/−	++	+++
BPH	I	15	0	20	80
	II	52	0	4	96
HGPIN	I	30	37	53	10
	II	5	0	40	60
PC (Gl. 6–7)	I	41	68	22	10
	II	17	88	12	0
PC (Gl. 8–10)	I	30	70	20	10
	II	28	85	15	0

Abbreviations: BPH, benign prostatic hyperplasia; Gl, Gleason score; GR, glucocorticoid receptor; HGPIN, high-grade prostatic intraepithelial neoplasia; PC, prostate carcinoma. GR immunostaining was analysed in two cohorts of patients from Northwestern University (I) and Russian Cancer Research Center (II). *GR intensity was evaluated by +/− to +++ scoring. The number of samples with indicated score is presented as percent to the total number of evaluated samples.

Generation of GR-expressing LNCaP cells

To study the effect of GR re-expression on PC cell growth and transformation, we generated LNCaP cells stably expressing GR cDNA tagged with V5-tag at C-terminus using the lentiviral system. For tracking, we co-infected LNCaP-GR cells with yellow fluorescent protein (YFP)-expressing lentivirus (Figure 2a). LNCaP cells infected with the empty vector (LNCaP-V) or with the YFP-expressing lentivirus (LNCaP-YFP) were used as a negative control. The level of GR in LNCaP-GR cells was comparable to the level of endogenous GR in DU145 and PC3 prostate cells (data not shown).

In the non-stimulated LNCaP-GR cells, GR was expressed mostly in cytoplasm and in some cells in the nuclei (Figure 2b). This result probably reflects the altered ratio between GR and chaperone proteins in these cells, allowing partial spontaneous translocation of overexpressed GR in response to glucocorticoids in the serum. Upon stimulation, with fluocinolone acetonide (FA), exogenous GR readily translocated into the nuclei in $\sim$90% cells (Figure 2c and d). As expected, the treatment of LNCaP-GR cells with FA activated the glucocorticoid-responsive TAT3.Luciferase and MMTV.Luciferase reporters (Figure 2e).

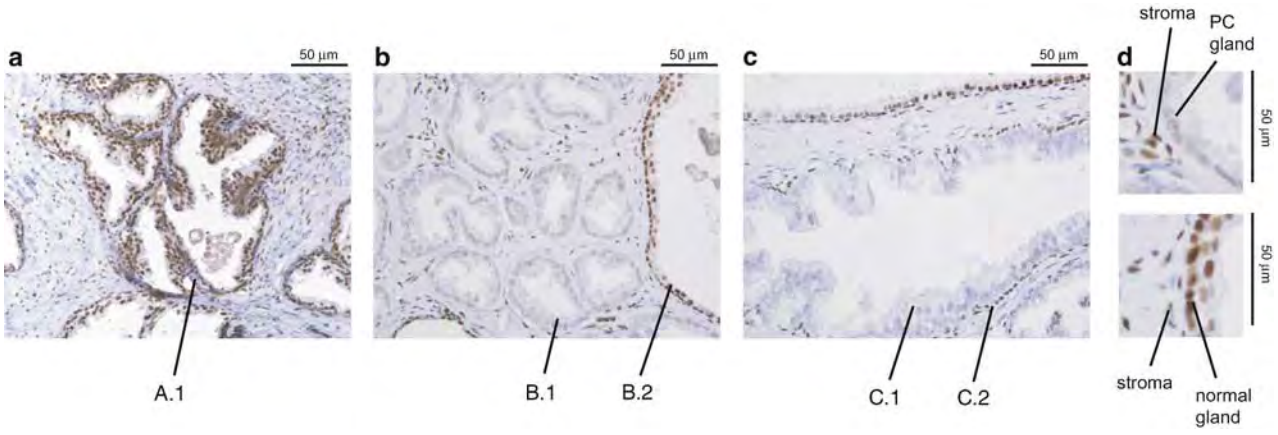


Figure 1 Expression of GR in BPH, PC and HGPIN. Immunolocalization of GR in paraffin sections of prostate tissues. (**a**) BPH; (**b**) PC (Gleason score 7); (**c**) HGPIN and (**d**) prostate stroma. Note: Low GR expression in PC (B1) and high GR expression in apparently normal prostate (B2) combined with positive nuclear GR staining in prostate stromal cells (**d**).

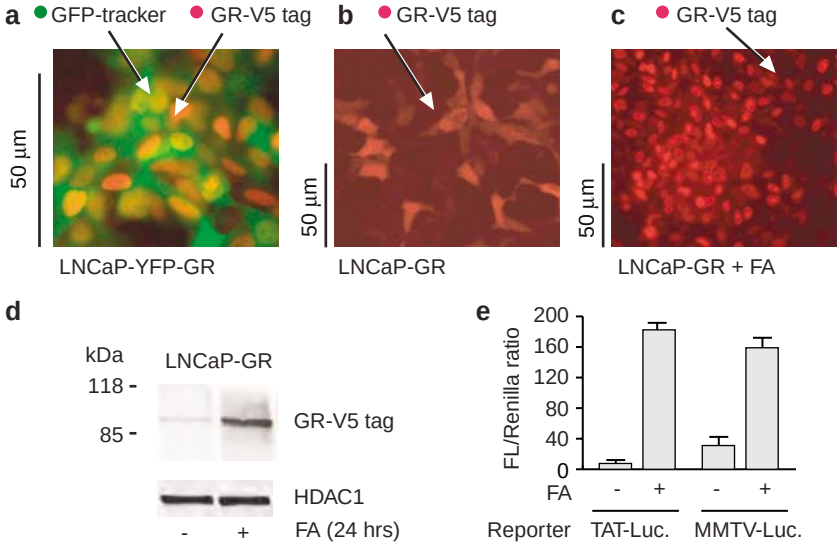


Figure 2 Characterization of GR in LNCaP-GR cells. (**a**) Monitoring of live LNCaP-GR cells. LNCaP-GR-V5 cells were co-infected with YFP-expressing lentivirus and stained with Ab against V5. (**b**–**d**) Glucocorticoid-induced nuclear translocation of GR. LNCaP-GR-V5 cells treated with vehicle (**b**) or FA, 10^{-7} M × 24 h (**c**) were stained with Ab against V5. (**d**) Western blot analysis of GR in nuclear protein extracts from control and FA-treated LNCaP-GR cells. (**e**) GR activity in dual Luciferase assay. FA-treated (10^{-7} M × 24 h) LNCaP-GR cells were transiently transfected with TAT- and MMTV-FL reporters, and RL reference reporter. FL activity was normalized against RL activity to equalize for transfection efficacy. The results of one representative experiment (three wells/experimental group) are shown as mean ± s.d.

GR signaling blocked proliferation and anchorage-independent growth but did not induce apoptosis in LNCaP-GR cells

We then studied the effect of restored GR signaling on LNCaP growth in monolayer and in soft agar. We took advantage of YFP expression in the LNCaP-GR-YFP cells to measure the actual number of cells/well. Glucocorticoid treatment of LNCaP-GR-YFP cells resulted in a strong growth inhibition (Figure 3a), whereas producing no significant effect on control LNCaP-V and LNCaP-YFP cells (data not shown).

On molecular level, the decreased proliferation was accompanied by upregulation of cyclin-dependent kinase inhibitors p21 and p27, decreased expression of cyclin D1 and proliferation marker Ki67, and a lower c-Myc phosphorylation (Figure 3c and d). Interestingly, the expression of p21 was increased in LNCaP-GR cells in comparison to LNCaP-V cells even without hormone treatment. This may be due to GR partial spontaneous nuclear translocation described above.

To assess the transformation levels *in vitro*, we measured anchorage-independent growth in soft agar. Even without FA, both the number and the size of the colonies formed by LNCaP-GR-YFP cells were decreased compared to the LNCaP-YFP control (data not shown). Upon glucocorticoid treatment, colony formation by LNCaP-GR-YFP cells was drastically decreased (Figure 3b).

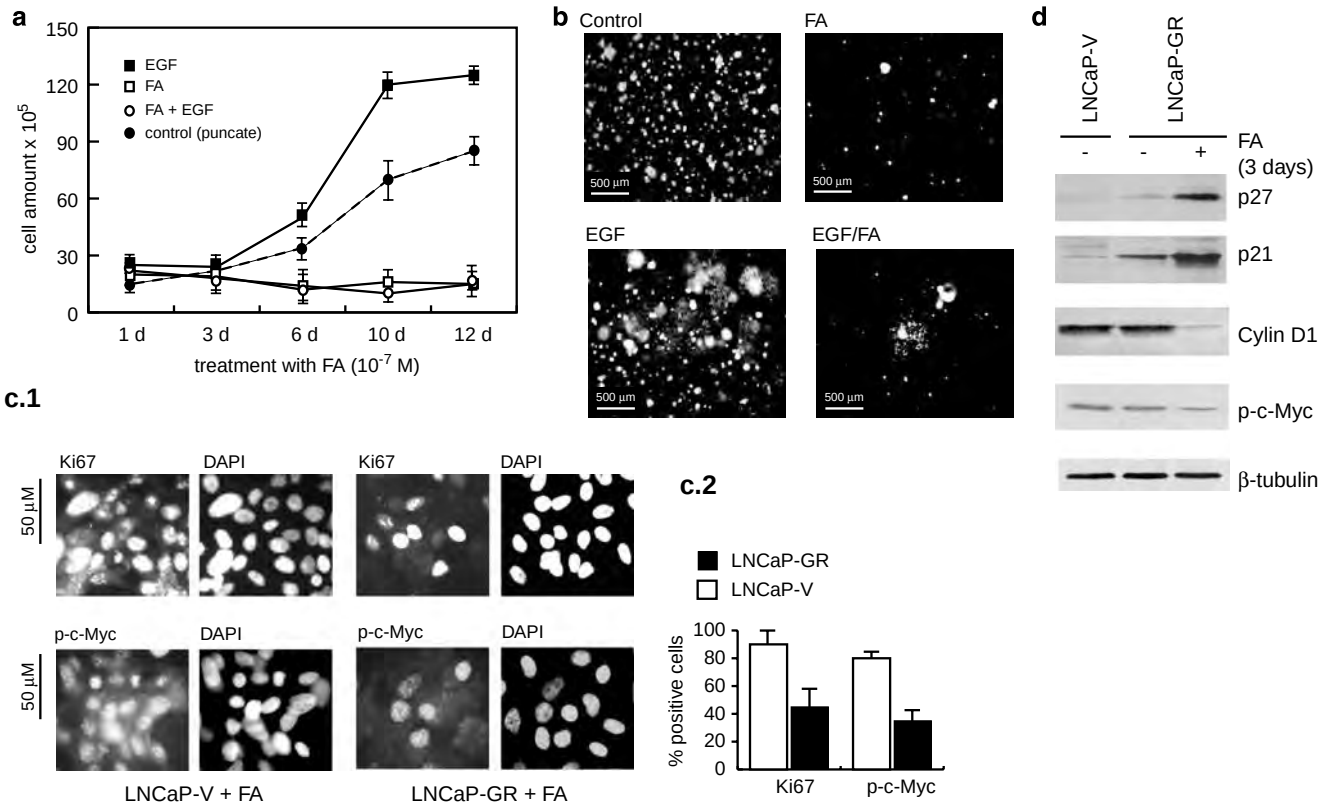


Figure 3 Inhibition of proliferation and anchorage-independent growth of LNCaP-GR-YFP cells by glucocorticoid. (**a**) Effect of FA on LNCaP-GR-YFP cell growth in monolayer. Number of LNCaP-GR-YFP cells treated with vehicle, FA (10^{-7} M), EGF (5 ng/ml) or EGF + FA was measured by YFP fluorescence using a plate reader. The results of one representative experiment (three wells/experimental group) are shown as mean ± s.d. (**b**) Effect of FA on anchorage-independent growth of LNCaP-GR-YFP cells. LNCaP-GR-YFP cells were grown in 0.6% soft agar for 2 weeks in the presence of FA (10^{-7} M), EGF (5 ng/ml), EGF + FA or vehicle (0.1% ethanol). (**c**) Immunocytochemical analysis of Ki67 proliferation marker and phosphorylated form of c-Myc. LNCaP-V and LNCaP-GR cells were treated with 10^{-7} M FA for 72 h. (c1) The immunocytochemistry results were quantitated as a percent of the positively stained cells to all cells (DAPI) in the field of view of microscope. Totally 500 cells were evaluated in each group. (**d**) Western blot analysis of cell cycle-related proteins. The expression of cyclin D1, p21, p27 and phosphorylation of c-Myc was evaluated by Western blotting in whole-cell protein extracts from LNCaP-YFP and LNCaP-GR-YFP cells treated with vehicle (−) and 10^{-7} M FA × 72 h (+).

As epidermal growth factor (EGF) signaling is important for PC growth and transition to the HRPC stage and triggers PC cell proliferation *in vitro* (Mimeault *et al*., 2003), we chose EGF as physiologically relevant stimulus to assess the GR effect on the induced PC cell growth. The recombinant EGF significantly augmented the growth of LNCaP-V and LNCaP-YFP cells (data not shown) as well as LNCaP-GR-YFP cells, both in monolayer and in soft agar (Figure 3a and b). The EGF effect was strongly inhibited by FA in LNCaP-GR-YFP cells (Figure 3a and b), but not in control cells (data not shown). Thus, activated GR strongly suppressed proliferation and anchorage-independent growth. This inhibitory effect was not attenuated by EGF, a well-known mitogen implicated in the progression of PC.

In some cell types including lymphocytes, glucocorticoid treatment may cause apoptosis (Bourcier *et al*., 2000; Greenstein *et al*., 2002). As shown in Figure 3a, FA significantly reduced the number of LNCaP cells on days 6–12 of the treatment. However, the analysis of the poly-(ADP-ribose) polypeptide (PARP) cleavage, mitochondrial potential and caspase activity in LNCaP-GR cells treated with FA did not reveal significant proapoptotic effect of glucocorticoids in PC cells (data not shown).

GR activation normalized the expression of PC markers

To further evaluate the effect of GR signaling, we investigated several early and medium/late PC markers whose expression typically changes during prostate tumorigenesis. For the profiling, we selected maspin that is usually downregulated in PCs, hepsin, which is upregulated in PCs and alpha-methylacyl-CoA racemase (AMACR) whose expression increases early, in both HGPIN and PC lesions (Chen *et al*., 2003; Ananthanarayanan *et al*., 2005). Western blot analysis and semiquantitative reverse transcriptase–polymerase chain reaction (RT–PCR) showed that in LNCaP-GR cells hepsin and AMACR were downregulated, whereas tumor suppressor maspin was upregulated upon FA treatment (Figure 4). Interestingly, the expression of PC marker genes was partially normalized in LNCaP-GR

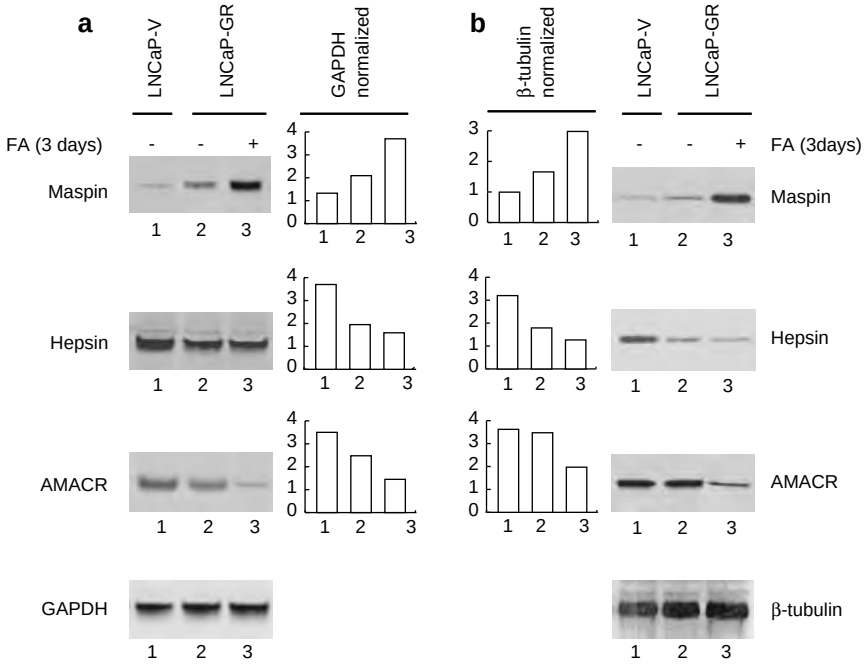


Figure 4 Effect of glucocorticoid FA on the expression of PC markers in LNCaP-GR cells. (**a**) Semiquantitative RT–PCR analysis of the PC markers expression. Total RNA from LNCaP-V and LNCaP-GR cells treated with vehicle (control) or with 10^{-7} M FA for 72 h was subjected to two-step RT–PCR. The amount of PCR products was measured and normalized to the amount of GAPDH PCR product. Quantitative data are presented as the ratio of GAPDH-normalized amount of PCR product in FA-treated vs vehicle-treated cells. (**b**) Western blot analysis of the PC markers expression. Protein expression was analysed in whole-cell protein extracts from LNCaP-V and LNCaP-GR cells treated with 10^{-7} M FA for 72. Signals were quantified as described in Materials and methods.

cells even without FA treatment. This could be attributed to the partial nuclear localization of GR in the untreated LNCaP-GR cells (see Figure 2). In summary, we conclude that the restoration of GR signaling resulted in overall normalization of PC cell phenotype.

GR activation blocked MAPK activity in LNCaP cells

The inhibition of MAPKs is an important regulatory mechanism by GR (Kassel *et al*., 2001; Bruna *et al*., 2003; Necela and Cidlowski, 2004). Therefore, we examined the GR effect on the basal and inducible activity of MAPKs dual-specificity mitogen-activated protein kinase 1 and 2 (Mek1/2), extracellular signal-regulated kinase 1 and 2 (Erk1/2), c-Jun NH_2-terminal kinase (JNK)/stress-activated protein kinase (SAPK) and p38 using Western blot analysis with antibodies (Abs) specific for the active, phosphorylated forms of the respective kinases.

The levels of MAPK expression and phosphorylation were not affected by glucocorticoid FA in vector transfected LNCaP cells resistant to the growth-inhibitory effect of glucocorticoids (data not shown). In contrast, the LNCaP-GR cells had much lower basal levels of phosphorylated forms of Mek1/2, p38 and JNK/SAPK (Figure 5a), again likely reflecting partial GR activation discussed above. FA treatment caused dramatic time-dependent decrease of MAPK phosphorylation in LNCaP-GR cells. The level of phospho-MAPKs phosphorylation was decreased by the second day of treatment and further diminished during 3–6 day course of FA treatment (Figure 5a). The relatively slow inhibition of MAPK phosphorylation by glucocorticoids is in line with the previous findings (Kassel *et al*., 2001; Greenberg *et al*., 2002).

Interestingly, our experiments revealed that glucocorticoids also reduced the total amount of MAPK proteins. Mek1/2, p38 and SAPK/JNK protein levels decreased after 24 h FA treatment, and remained at this level thereafter (Figure 5a). Semiquantitative RT–PCR analysis of Mek1, Mek2, Erk1, Erk2, p38 and JNK/SAPK has not revealed significant inhibition at the mRNA level (data not shown). Therefore, glucocorticoid treatment may have affected either translation or stability of MAPK proteins.

MAPK cascade is activated by growth factors including EGF, cytokines and stress (reviewed by Maroni *et al*., 2004). We investigated possible GR effect on induced MAPK phosphorylation using the inducers of specific MAPKs (Maroni *et al*., 2004). We used EGF for Mek1/2 and Erk1/2 activation, and interleukin (IL)-1 or tumor necrosis factorα (TNF)α for JNK/SAPK and p38 activation. IL-1 and EGF activated the corresponding MAPKs in LNCaP-V control cells within 5–15 min of treatment (Figure 5b and data not shown). In LNCaP-GR cells, the effect of studied inducers on Mek1/2 and SAPK/JNK was preserved, but the effect on Erk1/2 and p38 phosphorylation was either weak or absent even without hormone treatment (Figure 5b). FA

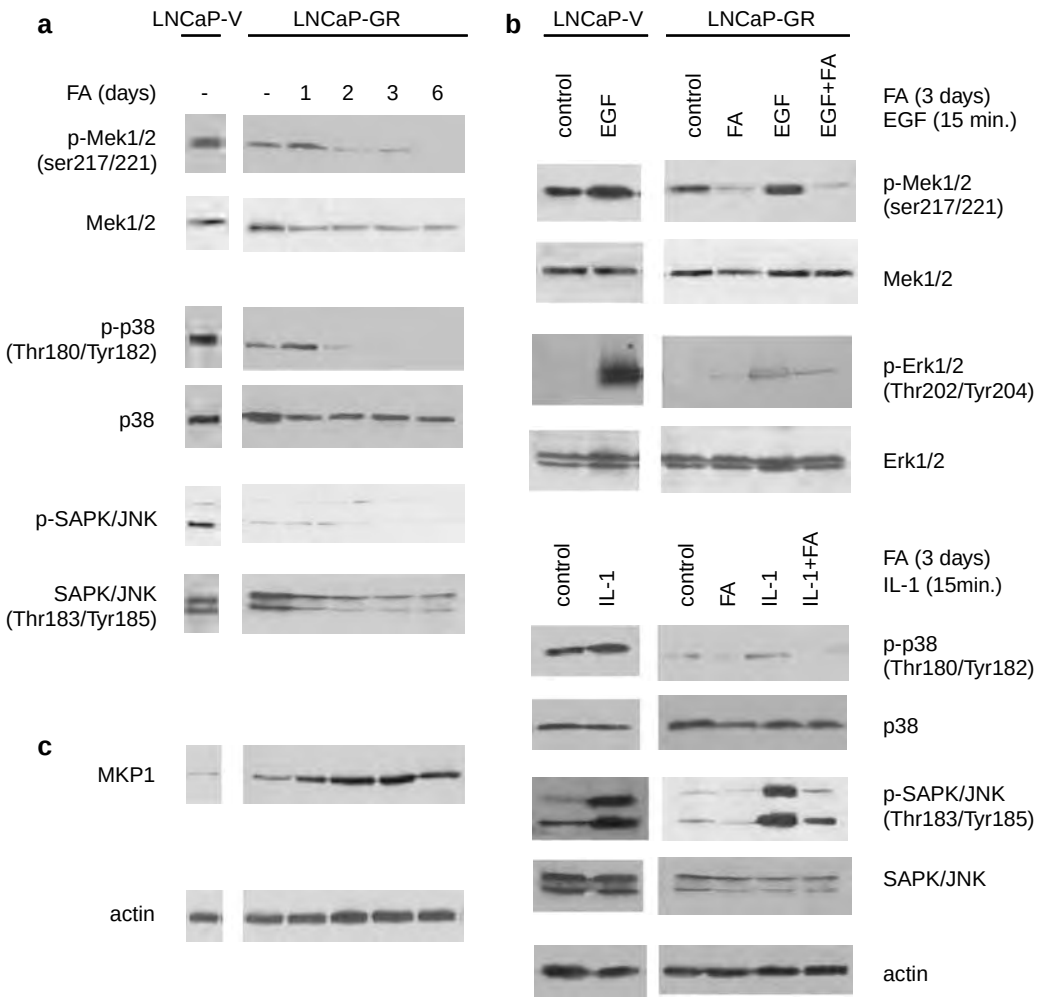


Figure 5 Inhibition of MAPK expression and activity by glucocorticoid in LNCaP-GR cells. MAPK expression and phosphorylation were analysed by Western blotting in whole-cell proteins extracts isolated from LNCaP-GR and LNCaP-V cells treated as indicated below. (**a**) Effect of FA on the constitutive MAPKs expression and activity. LNCaP-GR cells were treated with vehicle or 10^{-7} M FA for indicated time. (**b**) Effect of FA on the inducible MAPKs activity. LNCaP-GR and LNCaP-V cells were pretreated with 10^{-7} M FA for 3 days and MAPKs activity was induced by 15 min treatment with EGF (100 ng/ml); IL-1 (10 nM) or TNFα (10 ng/μl). (**c**) Effect of FA on MKP1. LNCaP-GR cells were treated with vehicle or 10^{-7} M FA for indicated time.

pretreatment decreased the effects of EGF and IL-1 on MAPK activation even further (Figure 5b). Similarly, p38 and SAPK/JNK phosphorylation upon TNFα treatment was also inhibited in LNCaP-GR cells pretreated with glucocorticoids (data not shown).

The negative effect of glucocorticoids on Erk1/2 and p38 phosphorylation is known to be associated with increased expression of MAPK phosphatase 1 (MKP1) (Kassel *et al*., 2001). We found that the expression of MKP1 protein was indeed increased in LNCaP-GR cells treated with FA (Figure 5c).

Overall our studies strongly suggest that GR tightly regulates both constitutive and inducible activity of multiple MAPKs in PC cells.

GR regulated TFs in PC cells

One important mechanism of gene regulation by GR involves its interaction with other TFs. We utilized novel protein–DNA array technology for simultaneous assessment of the DNA-binding activity of multiple TFs. The effect of GR on TFs was evaluated in LNCaP-GR cells after 3 days of FA treatment, the time point when MAPKs were strongly inhibited, but the effect on cell growth was modest. Vehicle-treated LNCaP-V cells were used as an additional control to evaluate constitutive DNA binding. Only reproducible $\geqslant$2-fold changes in DNA binding were pursued. We identified multiple TFs affected by activated GR in LNCaP cells (Figure 6a). Interestingly, $\sim$85% of those TFs were downregulated. GR-activated interferon γ activation site recognized by STAT3, and orphan nuclear receptor α. Conversely, GR inhibited several TFs known to interact directly with GR and involved in transrepression such as AP-1, NF-κB, CAAT/enhancer binding protein (C/EBP)α, activating transcription factor (ATF)-2 (CREB-BP1), p53 and SMAD3 (see references in Figure 6a). GR also blocked several TFs recognized as

a

TF name	1	2	3	Ref
Down-Regulated TF				
FRA-1/JUN AP-1 SRF	5.2 3.5 2.5	+ + +	SAPK/JNK Erk1/2	De Bosscher *et al*., 2003; Adcock, 2001; Necela and Cidlowski, 2004; Pulvere *et al*., 1991; Derijard *et al*., 1994; Inostroza *et al*., 2005; Karaganni *et al*.,1994
Ets-1 Elk-1	6.2 7.5	n/a n/a	Erk1/2, p38 SAPK/JNK	Bebied *et al*., 2003; Foulds *et al*., 2004; Ricote *et al*., 2006; Koul *et al*., 2004
STAT1/ISRE	3.1	+	Erk1/2	De Bosscher *et al*., 2003; Adcock, 2001; Necela and Cidlowski, 2004;, Xuan *et al*., 2005
C/EBPα	2.6	+	Erk1/2	Rudiger *et al*., 2002; Prusty *et al*., 2002
GATA4	3.7	n/a	Erk1/2	Liang *et al*., 2001
ATF2	3.6	+	Erk1/2, p38, JNK1/2	van Dam *et al*., 1995; Morton *et al*., 2004; Almlof *et al*., 1998
SMAD3	2.2	+	p38	Adcock, 2001; Song *et al*.,1999; Hayes *et al*., 2003
NF-κB	3.6	+	multiple	De Bosscher *et al*., 2003; Schacke *et al*., 2002; Adcock, 2001; Necela and Cidlowski, 2004
p53	3.7	+	n/a	Sengupta and Wasylyk, 2001
EGR1	2.8	n/a	Erk1/2 SAPK/JNK	Rolli-Derikinderen *et al*., 2003
PAX6	3.4	n/a	Erk1/2, p38	Mikkola *et al*., 1999
NFAT-c	2.8	+	Erk1/2 SAPK/JNK	Adcock, 2001; Porter *et al*., 2000; Lerner *et al*., 2003
Up-Regulated TF				
STAT3/GAS	2.3	+	Erk1/2 SAPK/JNK	De Bosscher *et al*., 2003; Adcock, 2001; Necela and Cidlowski, 2004; Lerner *et al*., 2003
RORα	3.1	n/a	n/a	

b

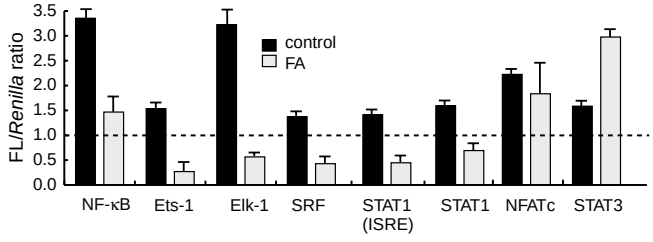
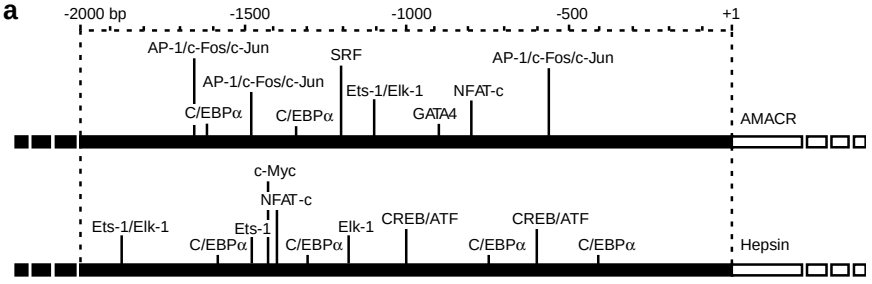


Figure 6 Analysis of glucocorticoid effect on TF activity in LNCaP-GR cells. (**a**) Analysis of multiple TF basal activity using Protein-DNA array. Nuclear protein extracts from vehicle-treated and FA-treated (10^{-7} M FA × 72 h) LNCaP-GR cells were used to analyse activity of multiple TFs (Protein-DNA interaction array, Panomics Inc., Fremont, CA, USA). (1) TF-DNA-binding change (folds of decrease or increase of DNA binding in FA-treated cells compared to control); (2) TF is a known partner for GR (+) and (3) TF is a known down-stream target for indicated MAPK. N/A – literature data are not available. See complete references in Supplementary material #1. (**b**) Validation of the Protein-DNA array. The array data were validated by transient transfections of LNCaP-GR cells with corresponding Luciferase reporter vectors and RL reporter. Cells were treated with vehicle or FA (10^{-7} M × 24 h). The transfection data are presented as described in Figure 2e. The results of one representative experiment (three wells/experimental group) are shown as mean ± s.d.

MAPK substrates/targets such as AP-1, SRF, Ets-1, Elk-1, STAT1, C/EBPα, GATA4, ATF2, nuclear factor of activated T cell (NFAT)-c, PAX6 and EGR1 (see references in Figure 6a of Supplementary material #1).

To validate the results of promoter array and to investigate the functional consequences of GR-induced changes in TF-DNA binding, we employed reporter assays. Reporter constructs for NF-κB, Ets-1, Elk-1, SRF, STAT1/ISRE, STAT1 and NFATc factors with Luciferase under promoters containing the appropriate binding sites (similar or identical to ones in TF array) were transfected into LNCaP-GR cells (Figure 6b). We



b **Hepsin**

TF	Position	Sequence
c-Ets-1	-1883	CCAGGAAG
	-1425	GCTTCCGGC
Elk-1	-1882	CAGGAAG
	-1169	GCAGGAACTG
C/EBPα	-1580	TGGGGCAAG
	-1296	AGCAAAC
	-797	CCCTCCTC
	-402	CCCTCCTC
CREB	-402	AGGnnnTACGCCA
ATF	-596	TGAGGTCAGGG
NFATc	-1383	TTTCC
c-Myc	-1419	CGCCCAGGTGGCC

AMACR

TF	Position	Sequence
c-Ets-1	-1112	GCAGGAAGCA
Elk-1	-1113	CAGGAAG
C/EBPα	-1323	ACTGGAAAT
	-1619	TTTGCAAAT
NFAT-c	-794	TTTTTCTCTT
GATA4	-883	AGATAT
AP-1/	-545	TGAGTTA
c-Fos/	-1642	GTGACTAA
c-Jun	-1491	TGACTAA
SRF	-1203	CCAAT

Figure 7 *In silico* analysis of putative TF binding sites in the promoters of PC markers. (**a**) Map of predicted TF binding sites of hepsin and AMACR promoters. 5′ Upstream promoter sequences relative to the transcription start sites between positions −2000 and +1 were analysed by online TESS. (**b**) Position and sequences of the predicted TF-DNA binding sites in PC marker promoters.

were able to confirm transrepression of all studied TFs, with the exception of NFATc.

To link our data on TF regulation by GR to the regulation of PC markers by GR/glucocorticoids, we screened promoter sequences of AMACR, hepsin and maspin (between positions −2000 and +1 from the transcription start sites) for the appropriate binding sites using online Transcription Element Search System (TESS) (Schug and Overton, 2005). We found that promoters of hepsin and AMACR contained binding sites for TFs inhibited by GR including C/EBP-α, Ets-1, Elk-1, NFATc, SRF and GATA4 (Figure 7).

Discussion

Despite the wide use of glucocorticoids for PC treatment, the changes of GR expression during prostate tumorigenesis and its role in the prostate cells remain unknown. Here, we developed a comprehensive picture of GR expression during prostate tumorigenesis. We found that GR expression was decreased or absent in 70–85% of PCs compared to apparently normal prostate or BPH. We also revealed that the decrease in GR expression occurs early in prostate tumorigenesis, at the stage of HGPIN. The early loss of GR expression in prostate tumorigenesis resembles changes reported for estrogen receptor β, an inhibitor of prostate growth (Fixemer *et al*., 2003). In contrast, the expression of other steroid hormone receptors either remains stable, like AR, or is increased, like estrogen receptor α and progesterone receptor (Fixemer *et al*., 2003; Torlakovic *et al*., 2005). These results combined with our *in vitro* data discussed below strongly suggest a tumor suppressor role for GR in the prostate. Remarkably, the loss of GR was specific only for the epithelial compartment of PCs. In benign and malignant prostate specimens alike, the stromal cells showed predominant nuclear localization of GR (Figure 1 and Mohler *et al*., 1996), suggesting an important role of GR specifically in prostate epithelium. It will be important to understand the molecular mechanisms that underlie the decrease of GR expression in PC cells. Even though the regulation of GR expression has not been well studied, the recent data indicate that DNA methylation is one of the mechanisms of epigenetic regulation of GR expression (Weaver *et al*., 2005).

We evaluated the effects of restored GR signaling in LNCaP cells lacking endogenous GR on proliferation, differentiation and transformation. The inhibition of LNCaP-GR cell growth by FA correlated with the decreased Ki67 and Cyclin D1 expression, the increased expression of cell cycle inhibitors $p21^{Cip1}$ and $p27^{Kip1}$, and the decreased c-Myc phosphorylation. Cell cycle-related proteins affected by glucocorticoids in LNCaP-GR cells are highly relevant to PC: cell cycle aberrations in PC are frequently linked to increased expression of cyclin D1 and other G1 phase cyclins, decrease in cell cycle inhibitor $p27^{Kip1}$, amplification and activation of c-Myc (reviewed by Quinn *et al*., 2005).

To evaluate the effect of GR signaling on LNCaP-GR cell differentiation, we analysed intermediate/late PC markers hepsin and maspin, and the early PC marker

AMACR, all currently introduced for routine PC diagnostics (Dhanasekaran *et al*., 2001; Chen *et al*., 2003; Jiang and Woda, 2004). All these markers are important for PC growth, angiogenesis and metastases. AMACR and hepsin are protumorigenic (Zha *et al*., 2003; Klezovitch *et al*., 2004), whereas maspin is an established tumor suppressor in different types of epithelium, including prostate. Maspin blocks angiogenesis, growth and invasion by PC cell *in vitro* and *in vivo* (reviewed by Schaefer and Zhang, 2003). We showed that GR-mediated signaling promotes differentiated state in PC cells where potential oncogenes hepsin and AMACR are downregulated and the expression of tumor suppressor maspin is increased. We also showed dramatic decrease of transformation reflected by the loss of anchorage-independent growth. In summary, we for the first time demonstrated overall normalization of PC cell phenotype by GR signaling.

Finally, we performed fine dissection of the mechanisms underlying GR antitumor activity in PC cells. One important mode of gene regulation by GR, transrepression, is in most cases mediated by direct interaction between GR and other TFs or by the crosstalk between GR and other signaling pathways, especially MAPKs (Kassel *et al*., 2001; Schacke *et al*., 2002; Bruna *et al*., 2003; De Bosscher *et al*., 2003; Necela and Cidlowski, 2004).

MAPK-mediated signaling is crucial for proliferation and survival of tumor cells (Greenberg *et al*., 2002; Ricote *et al*., 2006). Although changes in activation of specific MAPKs during prostate tumorigenesis are complex, nuclear expression of activated Erk and p38 and the level of phosphorylation of their targets Elk-1 and ATF-2 are frequently increased in PCs (Ricote *et al*., 2006; Gioeli *et al*., 2006). We found that activation of GR signaling blocked the activity of four major MAPKs: p38, JNK/SAPK, Mek1/2 and Erk1/2. These data are in line with the observations that glucocorticoids suppress MAPKs activity in some cell types. One of the mechanisms of GR inhibition of MAPKs involves increased expression of MKP1 (Kassel *et al*., 2001), a primary glucocorticoid-responsive gene (Wu *et al*., 2004). We also found the increased MPK1 expression in GR-positive cells treated with glucocorticoids. Our experiments also revealed an additional level of MAPK regulation by glucocorticoids, via post-transcriptional decrease of the total MAPK protein amount. Overall our results indicate that MAPKs are tightly regulated by GR/glucocorticoids in prostate cells.

Further, we showed that among numerous TFs whose activity was altered by GR in LNCaP cells, more than 85% were downregulated upon GR activation. Many of those, including AP-1, SRF, Ets-1, Elk-1, STAT1/ISRE, ATF2, C/EBPα, GATA4, EGR1 and PAX6 are recognized MAPK targets (references in Figure 6a and Supplementary material #1). Thus, their downregulation is an obvious consequence of the MAPK blockade by glucocorticoids. However, without further studies, we cannot rule out other mechanisms including the diminished expression of those TFs or their direct interaction with GR. Importantly, TFs repressed by GR contribute to the different steps of prostate tumorigenesis (references in Figure 6a and Supplementary material #1), and may control the expression of PC markers and differentiation of PC cells (Peterziel *et al*., 1999; Grossmann *et al*., 2001).

In summary, our results suggest that GR signaling has an antitumor effect in prostate cells, and that glucocorticoid treatment of patients at early stages of prostate tumor development such as HGPIN, when PC cells still express GR, may result in the inhibition of PC growth and normalization of PC cell phenotype. In the future, it will be important to extend our studies, and to evaluate the GR expression in prostate at the stage of HRPC and in PC metastatic lesions. In any case, the changes in GR expression should be taken into consideration to design the optimal time regimens for PC patient treatment with these steroid hormones and to enhance the clinical benefit of glucocorticoid therapy.

Materials and methods

Cell cultures and treatments

LNCaP cells (American Tissue Culture Collection, Rockville, MD, USA) were cultured in RPMI1640 medium (Gibco BRL Life Technologies, Rockville, MD, USA) with 10% FBS (HyClone, Logan, UT, USA), sodium pyruvate (10 mM), *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (10 mM) and antibiotics (Gibco BRL Life Technologies, Rockville, MD, USA) (referred thereafter as complete medium). The cells were treated with 10^{-9}–10^{-6} M FA (Sigma, Saint Louis, MO, USA), TNFα (10 ng/ml), IL-1 (1 μg/ml) and EGF (1–100 ng/ml) (all from BioSource Inc., Camarillo, CA, USA) where indicated.

Generation of LNCaP-GR cells

To generate LNCaP cells stably expressing rat GR cDNA (kindly provided by Dr M Beato, Philipps-Universitat, Marburg, Germany) tagged with V5 at the C-terminus, we used lentiviral system (Invitrogen Corp., Carlsbad, CA, USA). For selection of GR-positive clones, 6 μg/μl blasticidin was applied. For easier tracking, a second line of LNCaP cells was co-infected with CMV.GR-V5tag and YFP lentiviruses, and the cells containing YFP were selected by sorting. Control cell lines were established by infecting LNCaP cells with either the empty lentivirus (LNCaP-V) or the YFP-expressing lentivirus (LNCaP-YFP).

Western blot analysis

Whole-cell protein extracts were prepared using radioimmunoprecipitation assay buffer as described elsewhere (Rosenberg, 1996), resolved by sodium dodecyl sulfate–polyacrylamide gel electrophoresis on 10% gels, transferred to nitrocellulose membranes (BioRad, Hercules, CA, USA), incubated with primary Abs (see Supplementary material #2) followed by peroxidase-conjugated anti-rabbit or anti-mouse immunoglobulin (Ig)G secondary Abs (Cell Signaling Technology, Beverly, MA, USA) and ECL reagent (Amersham Pharmacia Biotech, Sweden) for the band visualization. To verify equal loading and adequate transfer, the membranes were probed with anti-actin and/or anti-β-tubulin Abs (Santa Cruz Biotechnology, Pasadena, CA, USA). To quantify the signals, images were scanned and digitized using ImageJ software (NIH, Bethesda, MD, USA).

Transient transfections and Luciferase assay
PC cells at 70% confluence were transfected with reporter Luciferase plasmids (see the list of reporter plasmids in the Supplementary material #3) in 24-well plates using Effectene reagent (Qiagen Inc., Valencia, CA, USA). Each well totally contained 0.2 μg of the plasmid DNA. All experimental and control groups contained at least three wells. The cells were harvested 36 h after transfection and Luciferase activity was measured using commercial Luciferase assay (Promega Corp., San Luis Obispo, CA, USA) on a TD20/20 Turner luminometer (Turner Design Instruments, Sunnyvale, CA, USA). When necessary, the cells were pretreated with 10^{-9}–10^{-6} M FA or vehicle (0.1% ethanol) for 2 days before transfections. The transfection efficacy was normalized using *Renilla* Luciferase (RL) under minimal promoter (Promega, Madison, WI, USA) to equalize for the transfections efficiency.

RT–PCR
A two-step RT–PCR reaction using reverse transcriptase murine leukemia virus-RT, random primers and PCR-Supermix (both from Invitrogen Corp., Carlsbad, CA, USA) with appropriate PCR primers was performed using total RNA isolated by the RNAeasy kit (Qiagen Inc., Valencia, CA, USA). The PCR primers (see the primer sequences in the Supplementary material #4) were designed using the PrimerBank database (http://pga.mgh.harvard.edu/primerbank/), RTPrimerDB Real Time PCR Primer and Probe Database (http://medgen.ugent.be/rtprimerdb/index.php) and Vector NTI software (Invitrogen Corp., Carlsbad, CA, USA).

PCR products were run on 1.5% agarose gels, the actual amount of PCR product was measured by Agilent 2001 Bioanalyzer and normalized to the amount of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) PCR product. The quantitative data are presented as the ratio of GAPDH-normalized amount of PCR product in FA-treated vs vehicle-treated cells.

Proliferation assay
The proliferation was measured by direct cell counts, or for the YFP-expressing cells fluorescence was measured by a Victor plate reader (Perkin-Elmer, Boston, MA, USA) at 436 nm excitation, and 480 nm emission wavelengths. For both tests, the cells plated at 10^4 cells/well onto 12-well plates were cultured in complete media with 6 μg/ml blasticidin in the presence of FA, EGF or vehicle (0.1% ethanol) for 1–12 days. Each experimental and control group consisted of three wells.

Colony formation assay in soft agar
The modification of previously described standard assay (Li and Johnson, 1998) was used. Briefly, the cells were trypsinized, washed in complete medium, resuspended in the medium with 0.6% agar and plated over the pre-formed agar underlayers (1% agar in complete medium) in 12-well plates (10^4 cells in 350 μl/well). After 2 and 4 weeks, the colonies were analysed using Zeiss fluorescent inverted microscope AxioVert. Each experimental and control group consisted of six wells.

Immunostaining of prostate tissues and cell cultures
Details of tissue collection procedure, immunostaining of cell cultures and tissue samples, and morphological evaluation are described in Supplementary material #5. Tissues were obtained from two cohorts of consented untreated patients (aged 40–82 years) by TURP (transurethral prostatic resection) or radical prostatectomy. Immunostaining of paraffin-embedded sections of formalin-fixed prostate samples was performed using primary mouse monoclonal anti-GR Abs (Novocastra, Norwell, MA, USA) followed by secondary anti-mouse IgG-reagent provided in the diaminobenzidine chromogen-based Envision + System-HRP kit (DakoCytomation, Carpinteria, CA, USA) and counterstained in Mayer's hematoxylin. The number of prostate epithelial cells with nuclear GR signal was evaluated by + to + + + scoring.

Immunostaining of cell cultures was performed on sterile coverslips. Cells were fixed, permeabilized and incubated with primary Abs (overnight at 4°C) followed by anti-rabbit donkey fluorescein isothiocyanate-conjugated and/or anti-mouse donkey Cy-3-conjugated secondary Abs (both from Jackson Immunoresearch, West Grove, PA, USA) and application of 4,6-diamidino-2-phenylindole (DAPI) (Vector Laboratories Inc., Burlingame, CA, USA) to identify the nuclei.

Transcription factor protein/DNA arrays
To simultaneously evaluate the activity of multiple TFs, we used Combo-Array version of TranSignal protein/DNA interaction array (Panomics Inc., Fremont, CA, USA) containing probes for binding sites for over 300 TFs (for detailed description see Jiang *et al*., 2004 and Supplementary material #6). The experiment was repeated three times. The differences in signal between FA- and vehicle-treated samples $\geqslant 2$ were considered statistically and biologically significant if they were revealed in all three experiments.

In silico *analysis of TF binding sites*
In silico promoter analysis of hepsin, maspin and AMACR was performed using 5′ upstream promoter sequences between positions −2000 and + 1 from the transcription start sites. The online Transcription Element Searching System TESS was used (Schug and Overton, 2005). Only TF binding sites displaying no variability from canonical sequences were selected for the analysis.

Statistical analysis
All experiments were repeated at least three times. Mean and s.d. values were calculated using Microsoft Excel software and compared using paired Student's *t*-test. A *P*-value of <0.05 was considered statistically significant.

Abbreviations

AMACR, alpha-methylacyl-CoA racemase; AP-1, activator protein 1; BPH, benign prostatic hyperplasia; Erk1/2, extracellular signal-regulated kinase 1 and 2; FA, fluocinolone acetonide; HGPIN, high-grade prostatic intraepithelial neoplasia; HRPC, hormone refractory prostate carcinoma; MAPKs, mitogen-activated protein kinases; Mek1/2, dual specificity mitogen-activated protein kinase 1 and 2; NF-κB, nuclear factor kappa-B; PC, prostate carcinoma; PIN, prostatic intraepithelial neoplasia; SAPK/JNK, stress-activated protein kinase/c-Jun-N-terminal kinase; TF, transcription factor; YFP, yellow fluorescent protein.

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Androgen receptor targets NFκB and TSP1 to suppress prostate tumor growth *in vivo*

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The androgen role in the maintenance of prostate epithelium is subject to conflicting opinions. While androgen ablation drives the regression of normal and cancerous prostate, testosterone may cause both proliferation and apoptosis. Several investigators note decreased proliferation and stronger response to chemotherapy of the prostate cancer cells stably expressing androgen receptor (AR), however no mechanistic explanation was offered. In this paper we demonstrate *in vivo* anti-tumor effect of the AR on prostate cancer growth and identify its molecular mediators. We analyzed the effect of AR on the tumorigenicity of prostate cancer cells. Unexpectedly, the AR-expressing cells formed tumors in male mice at a much lower rate than the AR-negative controls. Moreover, the AR-expressing tumors showed decreased vascularity and massive apoptosis. AR expression lowered the angiogenic potential of cancer cells, by increasing secretion of an anti-angiogenic protein, thrombospondin-1. AR activation caused a decrease in RelA, a subunit of the pro-survival transcription factor NFκB, reduced its nuclear localization and transcriptional activity. This, in turn, diminished the expression of its anti-apoptotic targets, Bcl-2 and IL-6. Increased apoptosis within AR-expressing tumors was likely due to the NFκB suppression, since it was restricted to the cells lacking nuclear (active) NFκB. Thus we for the first time identified combined decrease of NFκB and increased TSP1 as molecular events underlying the AR anti-tumor activity *in vivo*. Our data indicate that intermittent androgen ablation is preferable to continuous withdrawal, a standard treatment for early-stage prostate cancer.

Key words: prostate cancer; androgen receptor; NFκB; angiogenesis; apoptosis

Androgen withdrawal, common treatment for prostate cancer (PrCa), frequently leads to androgen independence.[1,2] Androgen binding to AR facilitates AR dimerization and binding to the androgen response element (ARE) CGTACAnnnTGTTCT and transcription. In addition, AR mediates nongenomic androgen effects, intracellular calcium flux and kinase activation.[3] In androgen-independent cell lines, AR may cause cell growth in the absence of ligand.[4] Unlawful AR activation can occur without steroids *via* surface receptors, like HER-2,[5] or by growth factors, like interleukin-6, oncostatin-M or bombesin.[6,7] AR gene amplification can also lead to increased transcriptional activity.[8] PTEN, a tumor suppressor, along with membrane protein caveolin dampen AR activity.[9,10] Thus AR can be active even in low androgen environment. AR mutations cluster in an area that defines AR protein interactions,[11–13] they are rare in local disease[14–16] but frequent in metastases where they enable binding with estradiols, glucocorticoids and anti-androgens[11,17] (reviewed in Ref. 17).

The role of androgens in cell survival and proliferation remains controversial. In androgen-sensitive LNCaP cells, physiologic levels of dihydroxytestosterone (DHT) fail to induce prostate-specific genes but enhance growth, possibly *via* Rb phosphorylation,[18] or *via* CDK2, CDK4 and p16 genes[19]; moreover AR blocking agents inhibit proliferation.[20,21] Blocking AR with antisense oligonucleotides, ribozymes, or Hsp90 hampers PrCa expansion.[11] At the same time, androgen may halt cell cycle *via* p27,[18] and facilitates differentiation,[22] AR expression in null PC-3 cells causes growth arrest, apoptosis and decreased invasion,[23–30] and in DU145 cells, growth arrest and differentiation.[31] Moreover, AR activation by mitogentic androgen doses sensitizes prostate cancer cells to the cytotoxic insult by taxanes.[32]

High microvascular density (MVD) in PrCa marks poor prognoses and metastases.[33] Testosterone stimulates endothelial proliferation and vascular regrowth (angiogenesis) after castration, however these may be secondary, due to hypoxia.[34,35] In culture, androgens stimulate angiogenic factors *via* HIF-1.[36] The loss of angiogenesis inhibitors in PrCa has been demonstrated,[33,37] however direct androgen suppression was only shown for pigment epithelial-derived factor (PEDF).[37] Conversely, thrombospondin-1 (TSP1) is decreased or lost in hormone refractory disease.[38]

NFκB transcription factor is highly active in PrCa due to hyperactive regulatory IκB kinase complex.[39] NFκB promotes proliferation and inhibits apoptosis *via* c-myc, cyclin D, IL-6 and Bcl-2, or by suppressing Bax.[40] Noteworthy, in PrCa AR status inversely correlates with NFκB activity.[25,41,42]

We analyzed how inducible AR affects the tumorigenicity of AR-null PC-3 cells. Unexpectedly, the AR(+) PC-3 cells became less tumorigenic on ambient testosterone background. Moreover, AR(+) tumors displayed low MVD and massive apoptosis. The diminished angiogenesis was due to elevated TSP1, while increased apoptosis may be due to dramatically decreased NFκB activity. AR expression lowered NFκB RelA, mRNA and protein, and reduced RelA activity and nuclear localization. This, in turn, dramatically decreased pro-survival Bcl-2 and IL-6. Thus we have shown the anti-tumor activity of AR *in vivo* and identified some of its mediators.

Material and methods

Cells

Bovine adrenal capillary endothelial cells (BAMVEC) were grown in MCDB131 (Sigma) with supplements (BioWhittacker). PC-3 were maintained in RPMI1640 (Invitrogen), 10% FBS and 1% Penicillin/Streptomycin. PC-3 cells expressing tetracycline

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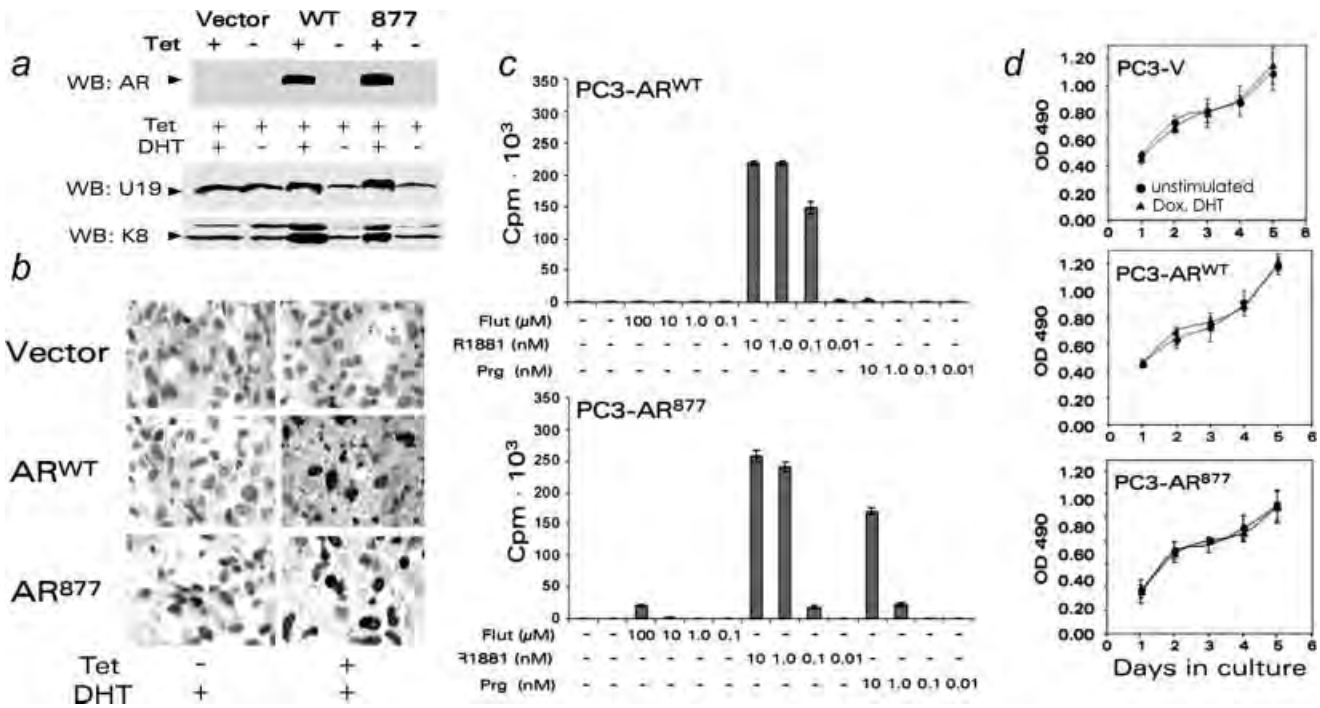

FIGURE 1 – Characterization of AR-expressing cells. (*a*) Inducible expression of AR and regulated genes. PC-3 clones expressing AR^{WT} and AR^{877} were treated with Dox and DHT, where shown, lysates resolved by SDS-PAGE and Western blots probed for AR (top), AR-dependent U19 (middle) and cytokeratin 8 (K8, bottom). (*b*) AR expression and localization was examined by IHC in similarly treated cells. (*c*) AR activity was tested with ARE reporter. Note AR^{877} activation by DHT (R1887), Flutamide (Fl) and Progesterone (Prg). (*d*) Growth curves of PC3-AR^{WT} and PC3-AR^{877} generated in media with DHT, ± Dox.

(tet) repressor (PC3-TR) were grown in tet-free serum (HyClone), and Blasticidin (1 μg/ml, Invitrogen).

To collect conditioned media (CM), 80% confluent cells were rinsed, incubated 48 hr in serum-free RPMI, media collected, cleared of debris, and concentrated in Millipore Ultrafree filters (5 kDa).

Cell growth was measured using WST-1 kit (Roche). The cells were plated in 96-well plates (5×10^2 cells/well), and induced with Doxycycline (Dox) (1 μg/ml, Fluka).

AR-inducible cells

We used T-REX inducible system (Invitrogen). The wild-type AR cDNA (Dr. X. Liao, University of Chicago, IL) and AR-877 mutant (Dr. Z. Culig, Innsbruck Medical University, Austria) were amplified, cloned into BamHI-Age I sites of pcDNA4/TO/myc-His vector and verified by sequencing. PC-3 cells were transfected with pcDNA6/TR (tet repressor) conferring Blasticidin resistance (FuGENE6, Roche). Transfectants were screened with β-gal reporter (pcDNA4/TO/lacZ, Invitrogen). PC3-TR cells were transfected with pcDNA4/TO/myc-His-AR. Cells resistant to Blasticidin/Zeocin were expanded and screened for AR expression. Clones with the lowest background expression were chosen (PC3-V, PC3-AR^{WT}, PC3-AR^{877}).

Western blotting

The cells were lyzed in PBS, 1% NP40, 0.5% Na deoxycholate, 0.1% SDS, and protease inhibitor cocktail (Sigma). Cleared lysates were resolved by SDS-PAGE and transferred to PVDF membranes. After blocking (5% Blotto in TBS-T, 20 mM TBS, pH7.4, 0.1% Tween-20) the membranes were probed and developed with ECL kit (Amersham). For IκB, total lysates were collected, resolved by SDS-PAGE, transferred to PVDF, blocked and probed in 0.5% BSA/TBS-T. For TSP1, CM (10 μg/lane) were resolved by 8% SDS-PAGE, membranes blocked in 7% Blotto and probed in 1% Blotto/PBS. For Bcl-2, membranes were blocked in 10% Blotto/TBS-T. The antibodies were: AR rabbit PAb (Ab-2, Santa Cruz), IκB-α rabbit PAb (Cell Signaling), TSP1 MAb (A4.1, Novus), Cytokeratin 8 pAb (Santa Cruz) and Bcl-2 antibodies (Santa Cruz). U19 antibodies were raised against GST-fusion protein and purified as described.[43]

IL-6 measurement

IL-6 was detected in conditioned media (CM) collected as above, using human IL-6 ELISA kit (BD Biosciences, San Diego, CA), as recommended by the manufacturer.

RT-PCR

RNA were extracted with GenElute kit (Sigma), converted to cDNA and amplified 30 cycles in 0.2 mM dNTPs, 1.5 mM $MgCl_2$, 0.1 μM primers and 1 U Taq polymerase (Fermentas); 2′ denaturation (94°C), 45″ annealing (55°C for actin, IL-6 and NFκB, 60°C for TSP1), 45″ elongation (72°C) with the following primers (5′-3′):

Actin, TGTTGGCGTACAGGTCTTTGC/GCTACGAGCTGCCTGACGG (182 bp);

TSP1, ACCGCATTCCAGAGTCTG/GACGTCCAACTCAGCATT (488 bp);

RelA, TATCAGTCAGCGCATCCAGACCAA/AGAGTTTCGGTTCACTCGGCAGAT (222 bp);

IL-6, AAGCCAGAGCTGTGCAGATGAGTA/AACAACAATCTGAGGTGCCCATGC (246 bp).

Luciferase assay

Cells were plated (3×10^5/well) in 6-well plates, induced 24 hr with 1.0 μg/ml Dox and transfected with 1 μg Firefly luciferase (FL) reporter, and 25 ng pRL-TK (Renilla luciferase, RL, Promega). R1881, (DHT), progesterone (Prg), flutamide (Fl) (Sigma) or vehicle (EtOH), were added for 24 hr. Luciferase activity was

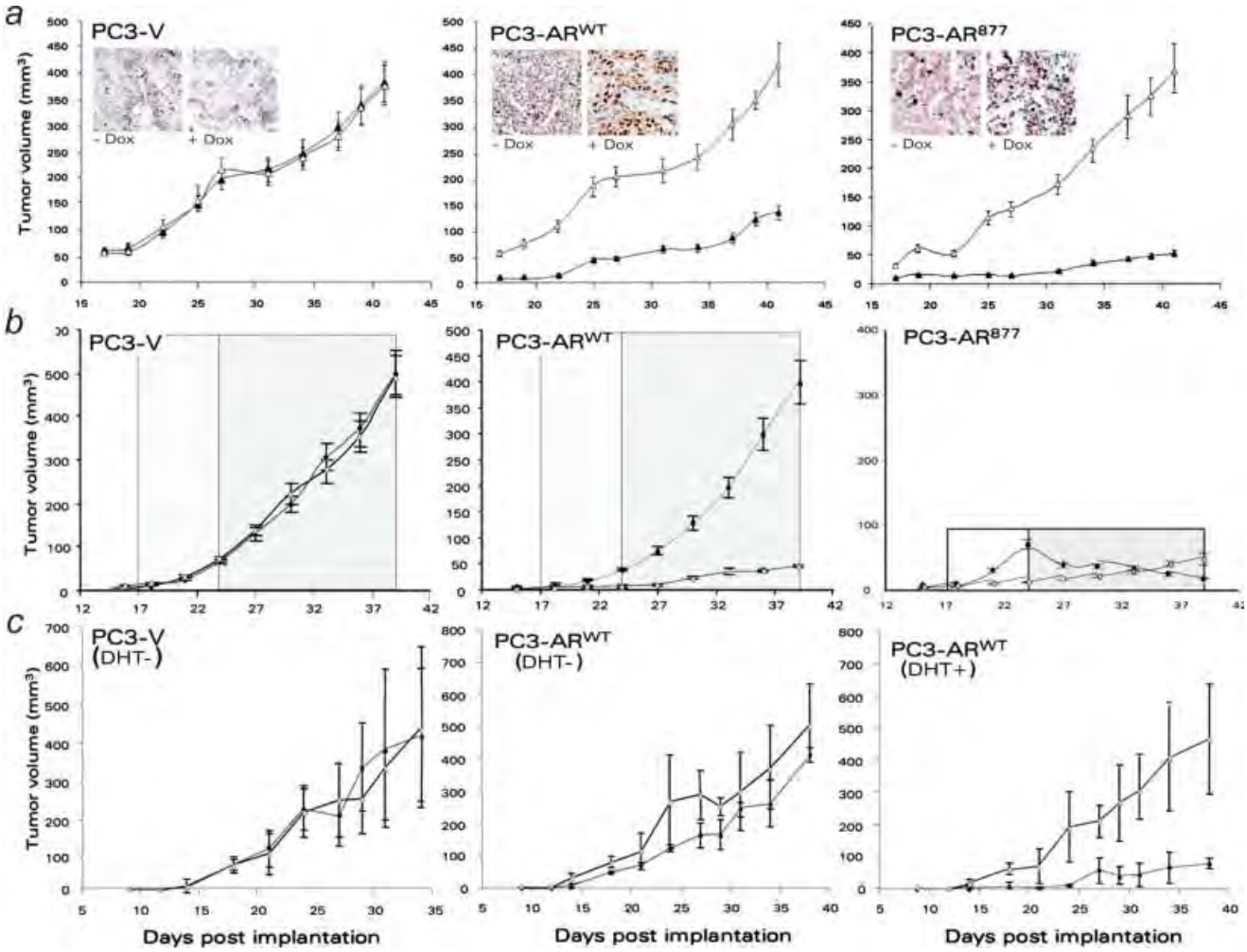

FIGURE 2 – The effect of inducible AR expression on the prostate carcinoma *in vivo*. (*a*) Male nude mice with flank injections of PC3-V, PC3-AR^{WT} and PC3-AR^{877} received Dox (▲) to induce AR expression, or plain drinking water (△). The expression and nuclear localization of AR has been confirmed by IHC (insets). Note delayed tumorigenesis by AR(+) cells. (*b*) Male nude mice were all treated with Dox and received flank injections of tumor cells as in (*a*). Half of the animals was treated with Fl (●), another half with vehicle (○). Note restored growth of PC3-AR^{WT} by Fl compared to the control and the lack of response to Fl by PC3-AR^{877}. (*c*) Female nude mice received flank injections of PC3-V and PC3-AR^{WT}, as in (*a*). The animals received Dox (●) or plain water (○). One half of the animals implanted with PC3-AR^{WT} were given DHT implants. Note the lack of growth inhibition by AR (Dox) in the absence of DHT and the delayed growth in Dox-treated animals bearing DHT implants.

measured using Dual Luciferase Reporter Assay (Promega). Luminiscence was assessed with Monolight 2010 Luminometer and the FL activity normalized against RL. Background (pGL3-TATA-Luc vector) was subtracted and fold induction calculated. The experiments were repeated in triplicate.

pGL3-TATA-Luc and AR reporter pGL3-GRE-Luc were form Dr. C. Kao, University of Indiana, Indianapolis. For TSP1 we used −2033/+150 promoter fragment[44] driving a RL reporter. The following AR/NFκB constructs were used: κB-FL reporter (5x κB promoter, Dr. WC.Greene, Gladstone Institute, UCSF); MMTV-FL reporter for steroid receptors, (Clontech, Palo Alto; CA); pcDNA3.1-CMV-p50 and pcDNA3.1-CMV-p65 (Dr. S. Okret, Karolinska Institutet, Sweden); pcDNA3.1-CMV-AR (Drs. O.A. Janne and J.J. Palvimo, University of Helsinki, Helsinki, Finland); and pcDNA-CMV-dnIκB-α (Dr. I.Verma, Salk Institute, La Jolla, CA).

For NFκB assays, 50% confluent PC-3 or LNCaP were transfected with indicated plasmids. After 36 hr the cells were harvested and Luciferase activity measured. Where shown, the cells were pre-treated 24 hr with DHT (Sigma).

Chromatin immunoprecipitation

Chromatin immunoprecipitation (ChIP) was performed using E-ZChIP kit (Upstate). Formaldehyde was added (final 1%, 10 min, 37°C), the cells washed in PBS, lyzed in 1% SDS, 10 mM EDTA, 50 mM Tris pH 8.1 and sonicated to produce ∼1 Kb DNA fragments. The samples diluted 1:10 in 0.01% SDS, 1.1% Triton X-100, 1.2 mM EDTA, 16.7 mM Tris-HCl, pH 8.1, 150 mM NaCl, were incubated with AR antibody (1:500, BD Biosciences). DNA/protein complexes were isolated on salmon sperm DNA agarose and extracted with 1% SDS, 0.1M $NaHCO_3$. Crosslinking was reversed, proteins digested with proteinase K and removed. DNA was precipitated, re-dissolved and amplified with TSP1 primers localized to the 1st intron (5′-3′): TGAGGCTTCAGTC-CCTCTGGT and AGTACAGACTCTTCCCTGAGTGCT (225 bp).

Migration assay

Migration assay was performed as in Ref. 45. BAMVECs starved in MCDB131, 0.1% BSA (Sigma), were plated at 1.5×10^6 ml^{-1} in Boyden chambers on the lower surface of gelatinized membranes (8 μm, Nucleopore). After attachment, serial dilutions

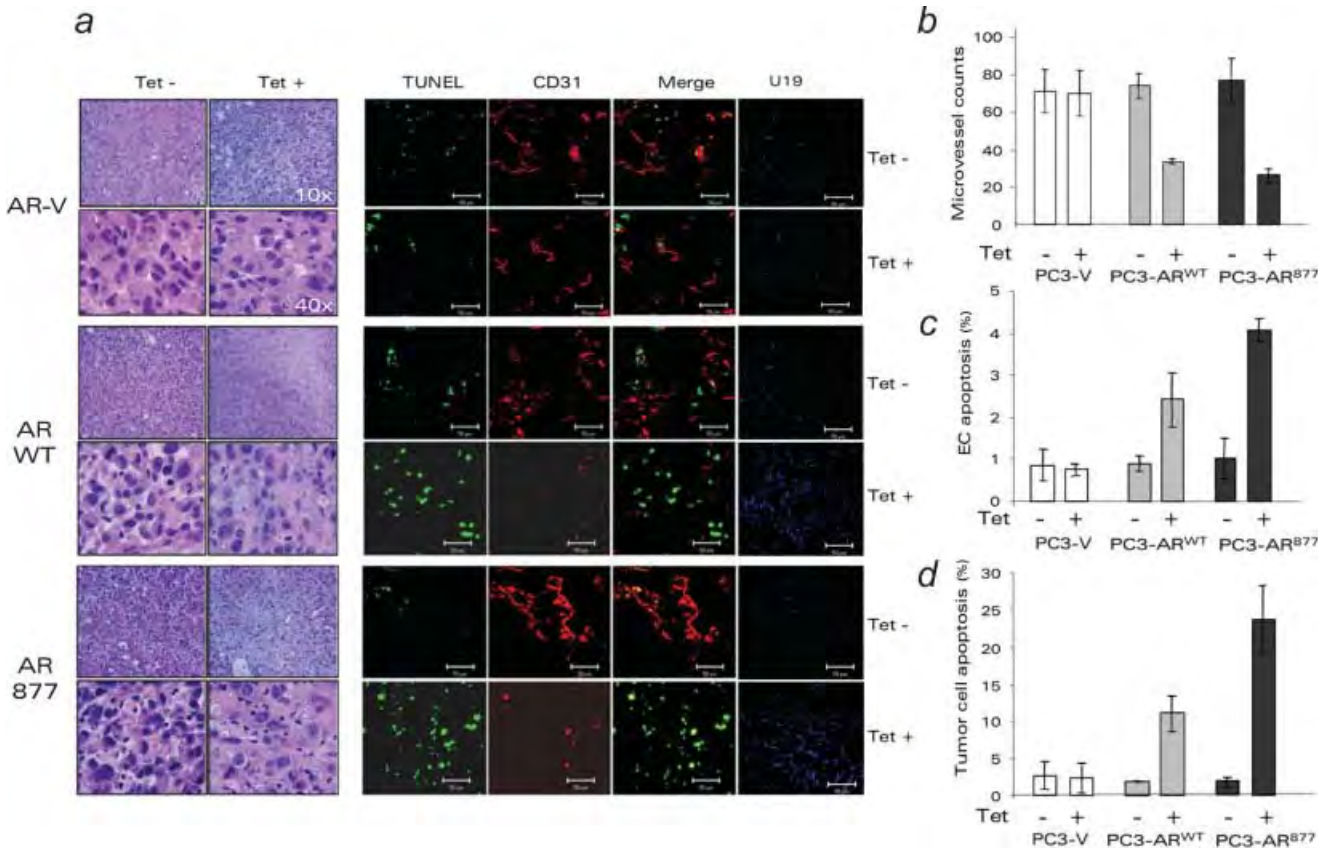

FIGURE 3 – AR-expressing tumors showed increased apoptosis and decreased MVD. (*a*) Paraffinized tumor sections were stained with H&E (left) and snap-frozen sections (right) for endothelial marker CD31 (red) and apoptosis (TUNEL, green). To confirm AR functional activity, the same sections were stained for U19 (blue). (*b*) Quantitative analysis of MVD. (*c*) Endothelial cell apoptosis calculated as percent of TUNEL-positive of total CD31-positive structures (merge, yellow). (*d*) Tumor cell apoptosis (total TUNEL-positive cells minus TUNEL positive endothelial cells). All measurements were performed with MetaMorph software.

of CM from PC3-V, PC3-AR^{WT} or PC3-AR^{877} were placed in top wells for 4 hr. Background migration (BSA) was subtracted and the data presented as percent maximal migration (10 ng/ml bFGF). All samples were tested in quadruplicate.

Tumorigenicity assay

PC3-V, PC3-AR^{WT} or PC3-AR^{877} cells were injected s.c. in hindquarters of athymic male mice (nu/nu, National Cancer Institute, 4–6 weeks), 10^6 cells/site, 5 animals/group, 2 sites/animal. To induce AR expression, Dox (1mg/ml) was given in drinking water. The tumors were measured every 3 days and the volumes calculated as length $\times$ width^2 $\times$ 0.52. The experiment was repeated using the same numbers of female athymic mice $\pm$ DHT pellets. The pellets were generated in the lab as described in Ref. 43. Flutamide (Fl) (40 mg/kg, Sigma) was given daily p.o. At the endpoint tumors were removed, snap-frozen or fixed in 4% formaldehyde. The animals were handled following the National Institute of Health guidelines, protocols approved by Northwestern University Animal Care and Use Committee.

Immunostaining

Five micrometer cryosections were fixed in cold acetone, 1:1 acetone/chloroform and acetone (10 min ea), rinsed in PBS, blocked with Avidin-Biotin Blocking kit, mouse Ig (Vector) and incubated 30 min with rat CD31 (1:125, PharMingen) and mouse TSP1 antibodies (1:100, Neomarkers). The slides were washed in PBS and incubated 15 min with donkey anti-rat RhodamineX antibodies (1:200, Jackson Immunoresearch) and biotinylated anti-mouse antibodies (1:200, Vector). Slides were developed with FITC-conjugated Avidin D (20 μg/ml, Vector). Biotinylated anti-rabbit antibodies were applied in blocking solution (1:200, 30 min) and followed by 1 μg/ml Streptavidin-Cy5 (Jackson Immunoresearch). To visualize apoptosis, the sections were evaluated by TUNEL (ApopTag kit, Serologicals).

For AR, 5 μm sections were deparaffinized, rehydrated, washed, antigen retrieved 15 min at 20–25 psi, 100°C in citric buffer pH 6.0 and 20 min at room temperature. Endogenous peroxidase was inhibited with blocking solution (Dako) and AR antibody added (30 min, N-20, Santa Cruz, 1:200) followed by HRP-conjugated anti-rabbit antibodies (30 min). Slides were developed with diaminobenzidine and counterstained with hematoxylin. Nonimmune rabbit serum served as negative control.

For NFκB, the sections after antigen retrieval were blocked with 20% goat serum in PBS, and incubated with mouse mAb for human p65/RelA (Cell Signaling), followed by fluorescent goat anti-mouse Ab (Jackson Immunoresearch). Representative experiments of 4 are shown.

Image quantification

Fluorescent images were obtained using Nikon fluorescent microscope (Diaphot 200) and converted to digital files using MetaMorph software. The same software was used to measure fluorescence intensity and compare the values to DAPI counterstain used as background. CD31-positive structures (MVD) were counted in 10 40$\times$ fields using MetaMorph software. Apoptotic

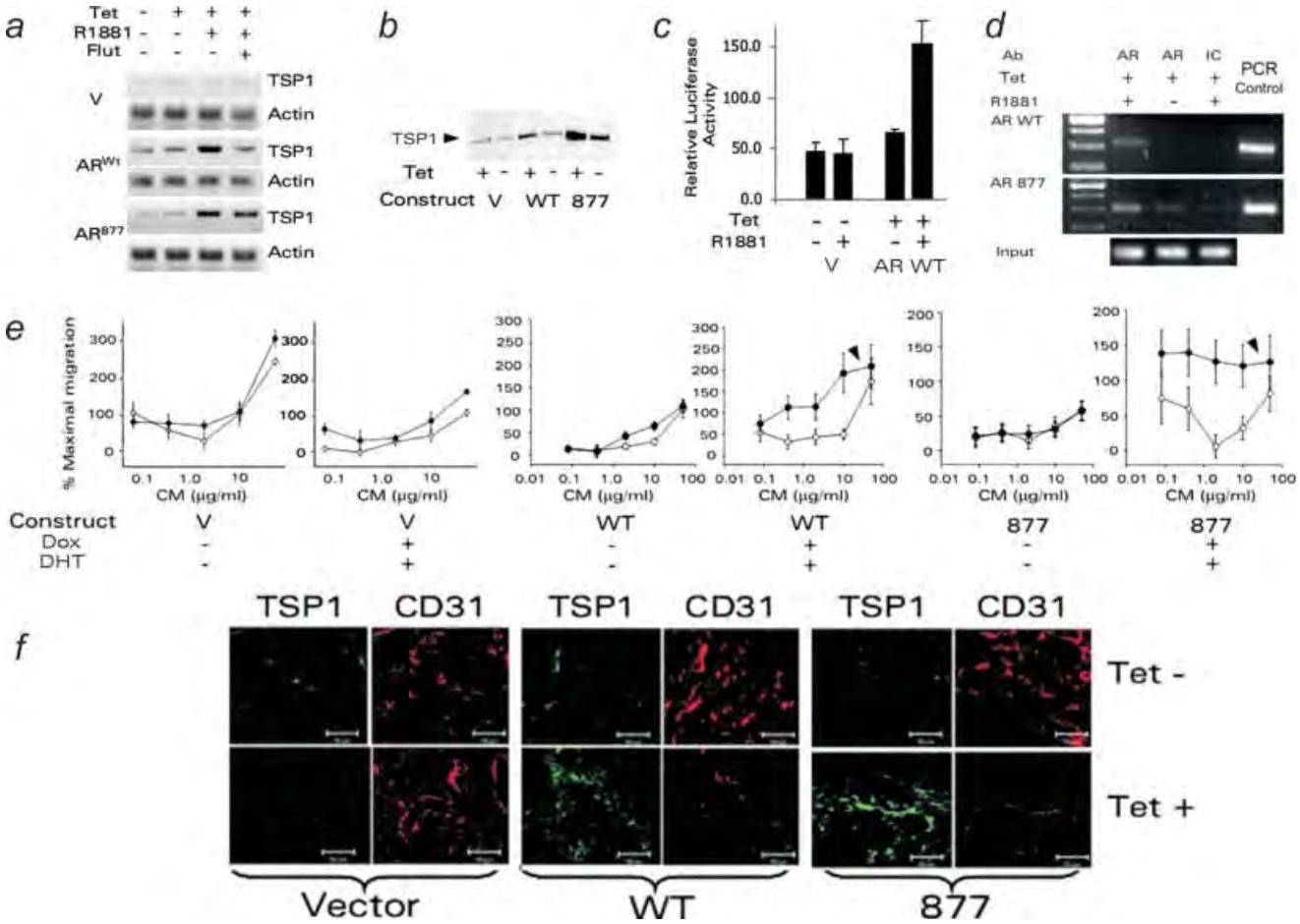

FIGURE 4 – AR decreased angiogenesis *via* angioinhibitory TSP1. (*a*) RT-PCR detection of TSP1 mRNA in PC3-V, PC3-AR^{WT} and PC3-AR.^{877} Note decreased TSP1 mRNA by Fl in PC3-AR^{WT} but not AR.^{877} (*b*) Western blot of Conditioned Media from PC3-V, PC3-AR^{WT} and PC3-AR^{877} ± Dox. All cells were DHT-stimulated. (*c*) The induction of TSP1-Luc reporter in AR(+) and control cells. (*d*) ChIP of the putative ARE in the 1st intron of TSP1 gene. IC: isotype control. PCR control: cloned TSP1 promoter amplified with the same primers. DNA input is shown. (*e*) Endothelial cell chemotaxis with the CM from PC3-V, PC3-AR^{WT} and PC3-AR$^{877.}$ The cells were treated with Dox and stimulated with DHT, where indicated and the CM collected and tested at increasing concentrations with the TSP1 neutralizing antibody (●) or with isotype control antibody (IgA) (○). Note the that CM from AR(+) cells are less potent at inducing endothelial cell chemotaxis, and that TSP1 neutralizing antibody, but not isotype control improves migration (arrowheads). (*f*) Immunostaining of the PC3-V, PC3-AR^{WT} and PC3-AR^{877} tumors in control and Dox-treated male mice. Cryosections were stained for CD31 (red) and TSP1 (green).

cells were quantified in 10 random fields using MetaMorph software.

Statistical analysis

Mean and standard error values were calculated and compared using paired Student's *t* test and ANOVA. p values < 0.05 were considered significant.

Results

AR induction reduced tumorigenicity

We generated PC-3 cells inducibly expressing wild-type AR (PC3-AR^{WT}) and promiscuous AR T877A[46] (PC3-AR^{877}) (Fig. 1*a*). Induced AR levels were comparable to LNCaP cells (not shown). The AR axis was restored and two AR-dependent genes, U19[43] and cytokeratin 8[47] robustly induced upon AR activation (Fig. 1*a*). Both wild-type and mutant AR became nuclear in the presence of DHT (Fig. 1*a*) and induced transcription of the ARE-luciferase reporter (Fig. 1*c*). AR877 was also activated by Flutamide and progesterone (Fig. 1*c*). The expression and nuclear localization of AR were induced *in vivo* in the PC-3 cells implanted in male mice upon Dox treatment (Fig. 2*a*, insets). However AR re-expression failed to enhance PC-3 growth in response to DHT (Fig. 1*d*). Moreover, AR(+) cells were less tumorigenic in male mice in the presence of Dox (Fig. 2*a*). When we used oral Flutamide to block endogenous testosterone, PC3-AR^{WT} regained tumorigenicity while PC3-AR^{877} did not (Fig. 2*b*), suggesting that weak activation by Fl was sufficient to suppress tumor growth. Finally, PC3-AR^{WT} cell formed tumors in Dox-treated female mice, obviously lacking endogenous testosterone, but not when they received DHT implants, underscoring the repression by androgen (Fig. 2*c*).

AR(+) PC-3 tumors had lower MVD and higher apoptosis rate

We measured MVD in the AR(+) and AR(−) PC-3 tumors. PC3-AR^{WT} and PC3-AR^{877} tumors in Dox-treated animals had 2.2–2.6 times lower MVD ($p < 0.01$) than untreated controls, or the AR(−) controls (Figs. 3*a* and 3*b*). TUNEL showed more endothelial and nonendothelial apoptotic cells in the AR(+) PC-3 tumors (Figs. 3*a*, 3*c* and 3*d*). AR remained functional in these tumors: its localization was predominantly nuclear in Dox treated

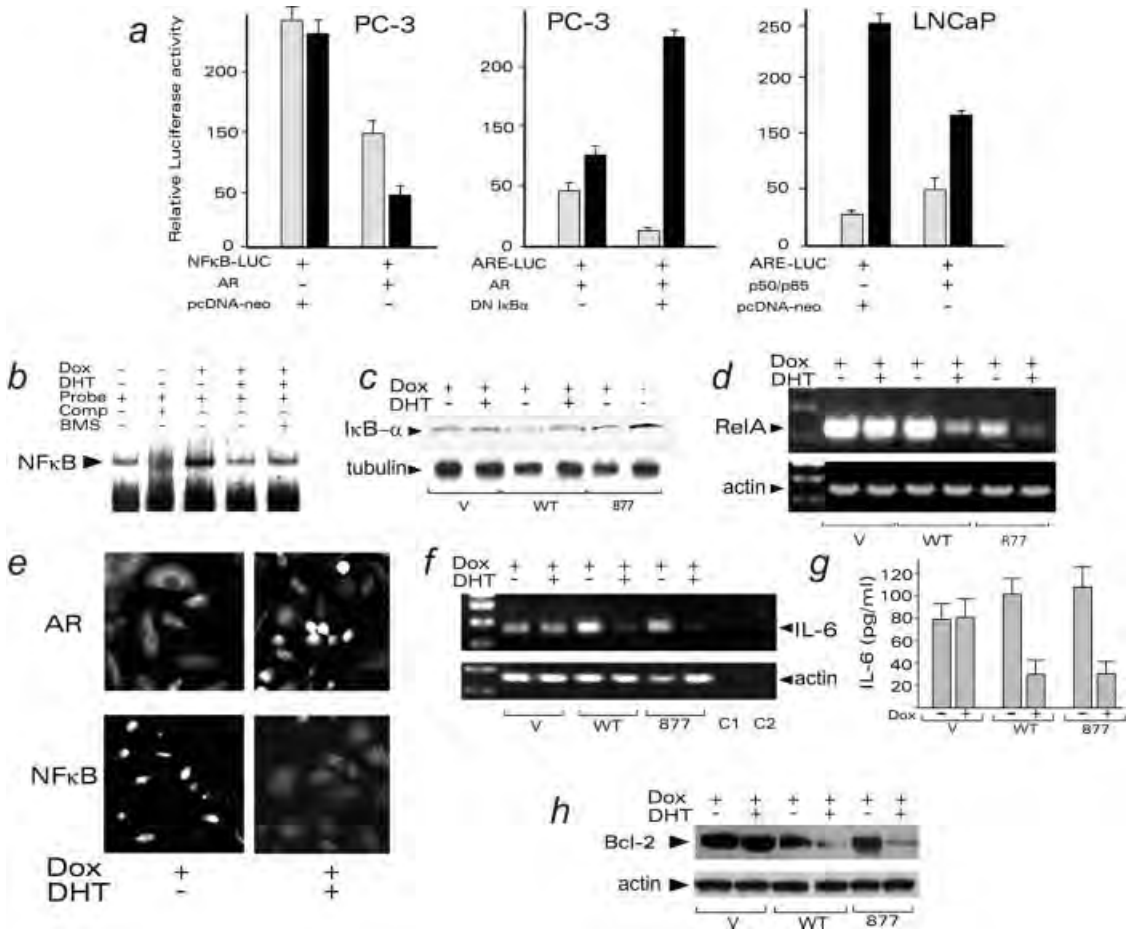

FIGURE 5 – The interference between AR and NFκB. (*a*) Reporter assays in cells transfected with the combination of NFκB-Luc, ARE-Luc, AR or p65/p50. pcDNA-neo was used to equalize the total DNA input. DHT (black bars) or control vehicle (ethanol: gray bars), where added, where indicated. Note decreased NFκB activity in the PC3-AR and increased AR activity in the presence of dnIκBα (NFκB superrepressor). (*b*) PC3-AR^{WT} cells were treated with Dox or Dox/DHT, nuclear extracts collected and analyzed by EMSA. Cold probe (Comp) or IKK inhibitor, BMS345543 (10 μM, BMS) added where indicated. (*c*) Total IκB-α in Dox/DHT stimulated cells. PC3-V -AR^{WT} and -AR^{877} cells were treated for 24 hours with vehicle ethanol or DHT, as indicated, and IκB-α detected in total cell lysates by Western blot. The blot was re-probed for β-tubulin to assess loading (lower panel). (*d*) RT-PCR of p65 (RelA) mRNA in similarly treated cells. Note a decrease upon AR activation. (*e*) PC3-AR^{WT} cells were grown on coverslips, treated as indicated and stained for AR (top) or NFκB (bottom). Note the lack of nuclear NFκB upon DHT stimulation. (*f*, *g*) IL-6 levels in Dox/DHT stimulated cells. The cells were treated as in (*c*), total RNA collected and RT-PCR performed with primers for IL-6 (F). C1, no cDNA; C2, no primers. (*g*) IL-6 detected by ELISA, in the media conditioned by similarly treated cells. (*h*) Western blot for Bcl-2.

males (Fig. 2*a*) and, AR responsive protein, U19 was strongly upregulated (Fig. 3*a*). Thus restoring AR axis in the androgen-insensitive cells delayed tumor progression, lowered MVD and increased apoptosis.

AR activation upregulated angioinhibitory TSP1

Seeking AR-dependent changes affecting MVD, we investigated angiogenic mediators in AR(+) and AR(−) cells. Three pro-angiogenic cytokines, VEGF, bFGF and IL-8, previously identified in PrCa,[36,48–53] remained unaltered. We were unable to detect changes in VEGF mRNA or protein using quantitative RT-PCR, ELISA, or immunostaining (data not shown). TSP1 is a critical angiogenesis inhibitor, whose expression is significantly lower in cancerous compared to the normal prostate[51,54]; an index integrating TSP1 with angiogenesis independently predicts survival.[52] In our model, TSP1 was low in parental PC-3 and PC3-V cells. In PC3-AR^{WT} and PC3-AR^{877}, TSP1 mRNA and secreted protein became high upon Dox/DHT stimulation (Figs. 4*a* and 4*b*). In PC3-AR^{WT} Dox/DHT increased activity of the luciferase reporter containing −2033/+150 TSP1 promoter fragment[44] (Fig. 4*c*). Moreover, ChIP demonstrated AR binding to the TSP1 promoter (Fig. 4*d*).

TSP1 suppressed angiogenesis in AR(+) cells

The migration of endothelial cells up the gradient of angiogenic factors is an important component of angiogenesis and an indicator of angiogenic activity of a given cell line.[55] The majority of natural inhibitors block endothelial cell chemotaxis induced by VEGF or by bFGF. To determine if TSP1 was responsible for the decrease of angiogenesis in AR(+) tumors, we examined endothelial cell chemotaxis to CM from the PC3-V and PC3-AR. PC3-V CM induced migration, with or without Dox and/or DHT, with EC_{50} = 2.4 μg/ml. CM from nonstimulated PC3-AR^{WT} and PC3-AR^{877} were also angiogenic, with similar EC_{50} (1.9–2.2 μg/ml), and not significantly altered by TSP1 antibodies (Fig. 4*e*). However, CM from PC3-AR^{WT} and PC3-AR^{877} stimulated to express AR and activated with DHT became less angiogenic (EC_{50} > 10 μg/ml). This lower angiogenic activity was due to TSP1, since TSP1 neutralizing antibody restored angiogenic activity (Fig. 4*e*). IHC showed MVD reduction in AR(+) tumors, paralleled by a

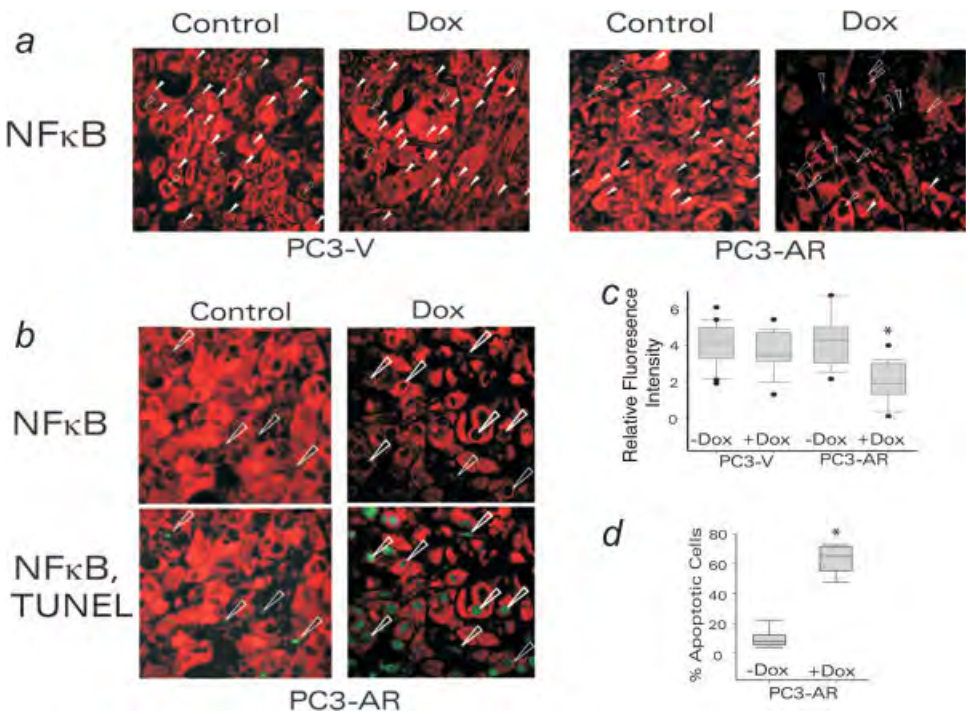

FIGURE 6 – AR effect on NFκB nuclear localization and apoptosis *in vivo*. (*a*, *c*) Immunostaining of AR(+) (PC3-AR) and control (PC3-V) tumors for p65 (RelA). The mice were given drinking water ± Dox, the resultant tumors stained for RelA (NFκB, red). (*a*) Representative images of the stained sections. Note nuclear RelA (solid arrowheads) in the absence of Dox and the lack of nuclear staining in Dox-treated tumors (empty arrowheads). (*c*) Immunofluorescence intensity was measured using MetaMorph software in a minimum of 12 random fields on 3 independent sections. (*b*, *d*) Inverse correlation between nuclear NFκB localization and apoptosis. Sections of Dox-treated or untreated (control) AR(+) tumors were stained for RelA (NFκB, red). Apoptosis was visualized by TUNEL (green). (*b*) Representative images of the stained sections. Upper panels show merged images. Empty arrowheads indicate cells lacking nuclear RelA. (*d*) TUNEL-positive cells were quantified in 6 random fields, 3 independent sections.

dramatic increase in TSP1 (Fig. 4*f*), pointing to a similar course of events *in vivo*.

AR activation lowered NFκB levels and activity

Seeking reasons for the decreased viability/increased apoptosis in AR(+) tumors we investigated NFκB status of AR(+) and (−) PC-3 populations. Constitutive NFκB activation and subsequent Bcl-2 increase mark hormone refractory PrCa.[42,56,57] Conversely, AR and NFκB counteract in transcription assays.[58] Indeed, reporter assays showed high basal NFκB activity in PC-3 cells, which was decreased upon transient transfection with AR^{WT} and diminished further by DHT (Fig. 5*a*, left). Moreover, ARE-Luc reporter activity, moderate in PC3 transfected with AR^{WT}, doubled in DHT-treated cells when NFκB was blocked with dnIκB-α (Fig. 5*a*, center). Conversely in AR-sensitive LNCaP p50/p65 dramatically reduced ARE-Luc transactivation, with or without DHT (Fig. 5*a*, right). EMSA showed that DHT significantly reduced NFκB DNA binding in PC3-AR cells (Fig. 5*b*). NFκB is chiefly regulated *via* cytoplasmic retention by IκB-α. However, the IκB-α levels in the AR(+) cells showed only modest increase, after Dox treatment (Fig. 5*c*) Unexpectedly, DHT significantly decreased the RelA mRNA in PC3-AR but not in PC3-V cells (Fig. 5*d*).

In addition, in PC3-AR^{WT} and AR^{877}, DHT lowered nuclear p65/RelA (Fig. 5*e* and data not shown). Nuclear localization of AR and p65 were mutually exclusive: in PC3-AR^{WT}, AR was predominantly cytoplasmic in the absence of DHT, while p65 was mostly nuclear. Conversely, in DHT-treated cells AR was predominantly nuclear, while p65 became cytoplasmic (Fig. 5*e*).

AR blocked pro-survival NFκB targets

DHT severely decreased the two NFκB targets, IL-6, as was measured at mRNA level and secreted protein (Figs. 5*f* and 5*g*), and Bcl2 (Fig. 5*h*). Both proteins are capable of increasing cell survival.

*AR diminished nuclear NFκB and increased apoptosis **in vivo***

The decrease in active NFκB remained true *in vivo*. While AR(−) tumors showed NFκB staining in the cytoplasm and nuclei, in AR(+) tumors RelA resided mainly in the cytoplasm (Fig. 6*a*). Similar to the *in vitro* results, RelA immunoreactivity was much weaker in AR(+) tumors (Figs. 6*a* and 6*c*). Higher incidence of nuclear NFκB was accompanied by low apoptosis rates, while in Dox-treated male mice AR(+) tumors showed less nuclear NFκB and higher apoptosis (Figs. 6*b* and 6*d*).

Discussion

Current *in vivo* models include tumor grafting in syngeneic or immune compromised animals, or autochthonous tumors in genetically manipulated mice. The differences in structure, physiology and cancer progression in mouse and human prostate[59] make it essential to complement the findings from genetically altered mice with those from xenografted tumors. Indeed, stroma and the smooth muscle are major structural and functional components in human, but not in mouse prostate. Lobular structure is seen in the mouse but not in human prostate, while mice have no transitional zone, prostatic urethra and capsule.[59] Most importantly, prostate cancer does not occur spontaneously in wild-type mice; the major-

ity of mouse models are driven by SV-40 large and small T viral oncogenes. Other suspect oncogenes and tumor suppressors yield intraepithelial neoplasia (PIN) but not PrCa.[60] Only three genes have been found critical for prostate carcinogenesis in mice: an oncogenic IGF-1 and cMyc, and a tumor suppressor PTEN.[61,62]

Surprisingly, AR failed as prostate-specific oncogene in transgenic models, its overexpression yields PIN but no invasive carcinoma.[63] In another study, wild-type and promiscuous AR mutant T857A (T877A analogue) fail to induce PIN in young animals suggesting that ligand driven AR activation does not induce epithelial hyperproliferation in the whole prostate.[64] Thus androgen role in PrCa is not unequivocal. In normal prostate it likey maintains homeostasis of proliferation *vs.* apoptosis, while androgen ablation changes AR targets from apoptotic to survival/proliferation. Interestingly, studies form Liao and coworkers demonstrate that AR-positive LNCaP cells, conditioned by long-term androgen withdrawal become hypersensitive to androgen and could be suppressed by androgen *in vivo*[65] and identify decreased cMyc and increased Bax as responsible genes.[66] Moreover. LNCaP sublines rendered androgen-independent, could be suppressed by androgen and then reversed to androgen-dependent phenotype.[67]

According to Greenberg and coworkers, transgenes encoding either AR-WT or AR-T857A, a mouse analog of human T877A mutant, did not cause prostate cancer in mice.[64] Consistent with their data, we showed that inducible wild-type and T877A AR failed to expedite tumor progression in a subcutaneous xenograft model, but instead caused dramatic delay in tumor progression, decreased MVD and increased apoptosis. Interestingly, these changes occurred predominantly *in vivo*. Other investigators observed decreased proliferation upon re-expression of AR,[26] however in our hands, AR(+) and (−) cells *in vitro* grew at the same rate. This difference may be due to the use of inducible AR expression, while stable transfectans may have acquired additional changes due to the constitutive AR overexpression. The molecular effects of AR expression/activation in PC-3 cells were twofold: decreased activation of NFκB, a pro-survival transcription factor in prostate epithelium,[40,56,57] and decreased overall angiogenic activity due to increased angioinhibitory TSP1, which translated into AR-dependent decrease of tumor MVD. The inverse correlation between TSP1 levels and prostate cancer progression and vascularization has been previously shown,[33,51,52,54] however TSP1 induction by AR has not been demonstrated.

The crosstalk between AR and NFκB has been previously shown *in vitro*, where NFκB inactivation resulted in higher apoptosis rates.[25,68] However, others indicate that AR also may increase NFκB activity.[69,70] Despite NFκB blockade, AR expression failed to increase apoptosis *in vitro*. Increased tumor apoptosis *in vivo* suggests that NFκB deactivation lowered the survival of AR(+) cells under stress. This is consistent with potentiated response to genotoxic stress by AR.[24] In our system AR(+) cells low in NFκB activity, become apoptotic in response to hypoxia due to insufficient angiogenesis. Conversely, AR(−) cells remain resistant. In addition, NFκB may contribute to the angiogenic properties of prostate epithelium by increasing NOS and cyclooxygenase-2[71]: its inactivation would further reduce tumor MVD.

It is widely accepted that functional AR is expressed in a large portion of advanced prostate cancers. However the majority of AR pathway genes (HERPUD1, STK39, DHCR24, and SOCS2) are suppressed in metastatic prostate cancer,[72] underscoring the fact that many of the AR targets counter cancer progression.

Our study indicates that both wild-type AR and AR with altered ligand specificity, lack the ability to transform prostate epithelium. Conversely, Greenberg and coworkers identified carcinogenic AR mutations in the transactivation domain.[73] Interestingly, somatic mutations associated with male infertility are in the DNA and lingand binding domains and the hinge, while ~40% cancer-associated mutations are in the transactivation domain, where they affect cofactor interactions. Although >80% AR point mutations have been identified in cancer specimens, their functional consequences are not verified, except for a few isolated cases. Combined data by Greenberg's group[64] and our's suggest that while AR maintains interactions with proper coactivators and corepressors, it continues to control homeostatic proliferation, apoptosis, and angiogenesis. One possible explanation is the release of AR control over NFκB activity: once disrupted, NFκB activation, in turn, favors increased survival, dampens stress responses and favors tumor progression. The mechanism of AR interference with NFκB remains unclear: although weak AR/NFκB interaction was observed *in vitro*,[74] the result has never been reproduced. Other investigators suggest competitive binding to adjacent cis-regulatory elements on the DNA.[75] We observed modest IκB-α increase in the AR(+) cells, however higher RelA mRNA and protein levels are more likely to play a role. Indeed, DHT stimulation on the AR(+) tumors produced the decrease in general NFκB immunoreactivity.

Our results suggest that persistent androgen ablation promotes the progression to androgen independent phenotype and indicate possible benefits of the treatment where androgen application and ablation are used in succession or intermittently.

Acknowledgements

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Appendix 3.

Yemelyanov A., Kobzeva V., Budunova I.
Role of IKKi in prostate cancer: A link between inflammation and androgen receptor signaling.
Proceedings of AACR, 2007 (abstract # LBA-9158).

Our recent data and data by others indicate that anti-apoptotic, pro-tumorigenic factor NF-kB is constitutively activated in androgen-independent prostate carcinoma (PC) cell lines and in prostate tumors. The important step in NF-kB activation is the phosphorylation of IkBa inhibitor proteins by IKK kinases (IKK) and IKK-related kinases IKKi/e and TBK1/NAK. IKKi is highly inducible kinase whose expression is known to be activated by numerous pro-inflammatory cytokines. We found that IKKi is expressed in androgen-independent PC cells (PC3 and DU145) with high level of constitutively active NF-kB but not in androgen-dependent PC cell lines (LNCaP and MDA PCa 2b) and primary prostate epithelial cells. Immunostaining revealed that IKKi is well expressed in BPH and PCs. Next, our data provide the evidence that IKKi could be involved in the regulation of NF-kB activity in PC cells through a positive feedback loop. Indeed, the treatment of PC cells with NF-kB inducers results in a rapid induction of IKKi. On the other hand, transient transfection of different PC cells with wild type (w.t.) IKKi results in activation of NF-kB. To further study the IKKi function in PC cells we generated PC3 and LNCaP cells stably expressing w.t. IKKi and dominant negative (d.n.) K38A mutant of IKKi using infection with corresponding lentiviruses. In both cell lines IKKi d.n. had no effect on cell morphology, growth, and tumorigenicity. In contrast, PC cells infected with w.t. IKKi displayed significant increase in growth in monolayer and in soft agar in comparison to cells infected with empty virus. As expected, stable overexpression of w.t. IKKi in PC cells resulted in NF-kB activation that correlated with increased level of phosphorylation of both IkBa (Ser36/32) and p65/RelA (Ser536). Unexpectedly expression of w.t. IKKi in LNCaP cells resulted in the increased expression of androgen receptor (AR) on both mRNA and protein level, and the accumulation of AR protein in the nucleus. We further demonstrated the increased basal activity of AR in LNCaP-IKKi w.t. cells, and the enhanced responsiveness to low doses of androgens. Experiments with androgen-deprived serum demonstrated that growth of LNCaP-IKKi w.t. cells was less dependent on the androgens than LNCaP-V cells. The mechanisms of regulation of AR expression and function by IKKi are currently under study. In conclusion, the revealed control of AR-mediated signaling by IKKi may represent an important regulatory link between the inflammation and tumorigenesis in prostate.

Appendix 4.

ROLE OF IKKs AND TRANSCRIPTION FACTOR NF-kB IN PROSTATE TUMORIGENESIS

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IMPACT DOD meeting, 2007, Atlanta. P. 118.

One of the contributing factors to high mortality rate from prostate cancer (PC) is the extreme resistance of PC cells to apoptosis induced by radio- and chemotherapy. Thus, the specific induction of apoptosis is an important target for PC treatment. One of the central anti-apoptotic pathways in cells is mediated by NF-κB transcription factor. NF-κB activation requires degradation of IκB inhibitory proteins which involves several steps including IκB phosphorylation by IκB kinases IKKα/IKKβ. Novel family of IKK-related kinases including inducible IKKi/ε activated by numerous pro-inflammatory cytokines, also could phosphorylate IkB and function further upstream in the NF-κB signaling pathway.

Using funds from DOD Prostate Cancer Research Programs (New Investigator award DAMD17-01-1-0015 and Idea Development award DAMD17-03-1-0522), we determined the status and function of NF-κB in PC cells and PC tumors. We found that NF-κB is constitutively activated in human androgen-independent PC cell lines and in human PCs (Gasparian et al., 2002). We also discovered that the level of IκBα phosphorylation and the rate of IκBα degradation are increased in androgen-independent malignant PC cells in part due to the high level of constitutive IKKα/IKKβ activity. Recently we found that IKKα/β are phosphorylated in PC samples suggesting that IKK kinases are indeed constitutively activated in prostate tumors (Yemelyanov et al., 2006).

Further, we discovered that IKKi is highly expressed only in androgen-independent PC cells with constitutively active NF-κB, and could be involved in the regulation of NF-κB activity in PC cells through a positive feedback loop (Yemelyanov et al., 2004). The forced expression of exogenous IKKi in LNCaP cells that lack this kinase, resulted in significant increase in NF-kB activity, and increased PC cell growth and tumorigenicity.

This work has been expanded towards the search for most effective strategies of NF-kB blockage in PC cells. We tested several novel compounds including highly specific IKKβ inhibitor PS1145, proteasomal inhibitor PS341/Velcade (both in collaboration with Millenium Pharmaceuticals Inc. , Cambridge, MA), as well as dissociated ligands of glucocorticoid receptor that inhibit NF-kB via stimulation of negative protein/protein interaction between activated GR and p65: AL438 (in collaboration with Ligand Pharmaceuticals, San Diego, CA) and Compound A (P.I. initiated research). We determined that IKKβ inhibitor PS1145 induced apoptosis in PC cells, inhibited their growth, and strongly inhibited their invasion (Yemelyanov et al., 2006). We also found that dissociated ligands of GR are highly cytostatic and cytotoxic in malignant PC cells (Yemelyanov et al., submitted). Finally, we showed that Selenium, an effective preventive agent for PC, inhibited NF-κB activity in PC cells, and that NF-κB blockage made PC cells more sensitive to Se-induced apoptosis (Gasparian et al., 2002).